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Epigenetic effects of matrix attachment regions on transgene expression in a maize callus line

by

Cory Ray Brouwer

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ABSTRACT

Matrix attachment regions (MARs) are DNA sequences that bind non-histone proteins in the chromatin scaffold and define discrete loops of DNA containing expressed genes *in vivo*. I have been studying the effects of flanking transgenes with MARs on transgene expression levels in BMS maize callus. Three MAR elements, two from maize (*Adh1* 5' MAR and *Mha1* 5' MAR) and one from yeast (ARS1), have very different effects on transgene expression that bear no relation to their affinity for the nuclear matrix *in vitro*. The main effect of MARs is to prevent silencing of some (but not all) transgenes, at least when transgene silencing is operative. Additionally, MARs can influence the establishment and heritability of transgene transcription states. During a period of five years, I found that the degree of transgene silencing in BMS cultures was progressively lost. The protective effect of MARs against transgene silencing was also progressively lost, suggesting that *trans*-acting factors responsible for both effects were gradually lost as the cells aged. This observation is very similar to observations made in yeast where SIR proteins that are responsible for establishment and heritability of gene silencing are lost or rendered functionally inactive as cells age. In yeast, SIR proteins also interact with the machinery responsible for non-homologous recombination (i.e. end-joining), and loss of SIR function is associated with reduced end-joining. Similarly, as transgene silencing was lost in BMS cultures, the levels of end-joining (as measured by stable transformation frequencies) also decreased. Thus, factors involved in cellular aging, transgene silencing, end-joining, and the *in vivo* effects of matrix attachment regions in a multicellular eukaryotic system may be inter-related, much as they are in yeast.

CHAPTER I. GENERAL INTRODUCTION

1.1 Research Problem

Targets for improvement in agriculturally important crops include disease and stress resistance genes or specialty traits that add value to the end-user. Until recently, new traits had to be introduced into elite parent lines through breeding programs that typically took many years. Plant transformation has shortened the time it takes to get a new trait into the desired genetic background. However, the introduction of transgenes into agriculturally important crop plants such as maize is still inefficient. Frequently, when a gene is transferred into maize or other plants by transformation, it is either silenced or subject to position effects. This makes it necessary to produce large numbers of transformants to generate an event with the desired characteristics. Development of methods to prevent silencing or position effects would greatly reduce the numbers of transgenic events that need to be generated and screened, thereby improving transformation efficiency.

1.2 Background and Significance

Prior to the start of this work, there had been several reports, in which MAR elements flanking transgenes appeared to result in increased transgene expression, reduction of position effects or copy number dependent expression. The original observations were made in mouse and chicken cell lines (Stief et al., 1989; Klehr et al., 1991), but similar data were reported in plant cell lines shortly thereafter (Breyne et al., 1992; Allen et al., 1993). Breyne et al. (1992) found that in transformed tobacco calli, a heterologous MAR derived from the human β-globin gene increased the average transgene expression level by

1.4-fold, but did not reduce position effects. In contrast, flanking transgenes with a plant MAR isolated from near the soybean lectin gene was able to reduce position effect variation in transgene expression levels by 60 percent. In another report, Allen et al. (1993) reported a 24-fold increase in transgene expression levels, but essentially no reductions in position effects. Thus, the effects of flanking transgenes with MARs appeared to be highly variable, and I suspected that more systematic study with larger sample numbers might help elucidate the underlying basis of this variability. Furthermore, I wanted to investigate the stability and predictability of MAR effects on transgene expression, to see if these interactions were genetic or epigenetic in nature.

To investigate the effects of flanking transgenes with MARs in maize, I chose to use Black Mexican Sweet (BMS) corn suspension cells, because they could be transformed at high frequency using particle gun bombardment. A yeast sequence (ARS1) reported to have MAR activity and effects on transgene expression in tobacco (Allen et al., 1993) was evaluated, alongside two MAR elements associated with endogenous maize genes (Avramova and Bennetzen, 1993).

1.3 Organization of Dissertation

Chapter II is a literature review. This review covers research on MAR elements from the discovery of the nuclear matrix to more recent work examining the effects of MARs in transgenic studies.

Chapter III describes materials and methods used in this study. The primary methods used in this research study involved plasmid vector construction, BMS cell transformation, gene expression assays, and statistical analysis of data.

Chapter IV describes and discusses the results of my work. This chapter is split into three sections. In the first section, I describe the effects of two MARs from maize and one from yeast on 35S::luciferase in BMS. I found that each MAR had different effects on transgene expression, that did not correlate with the binding affinity of each MAR for the nuclear matrix *in vitro*. By sampling large numbers of transformants, I also found that the distributions of transgene expression levels in BMS were bimodal and not normal.

In the second section, the effects of flanking two different transgenes with *Adh1* 5' MAR elements were compared. Because this study was done over a period of 5 years I was able to evaluate whether the effects of MARs on transgene expression were stable over time in different subcultures of the same BMS cell line. At the start of this study, I found that the *Adh1* 5' MAR prevented silencing of 35S::luciferase transgenes, but not 35S::*GUS*. As the BMS cells aged over time, the effects of the *Adh1* 5' MAR were masked by a progressive loss of transgene silencing. Thus, the outcome of flanking transgenes by MARs is neither stable nor predictable, and their effects are epigenetic. Progressive loss of transgene silencing is also seen in aging yeast cells, and our observations suggest that this may be a general property of eukaryotic cells, as they get older. My data also indicate that the same MAR sequence can affect both the establishment and maintenance or heritability of stable transgene expression states. The latter effect was inferred from the novel discov-

ery that derepression of silenced transgenes by a demethylating agent *in vivo* can be slowed by flanking MARs.

The final section of chapter IV describes a variety of other experiments investigating the effects of MARs on transgene expression, e. g. whether the size of the loop flanked by MAR elements or linearization of plasmid-borne MAR-flanked cassettes in the vector backbone makes any difference. Other experiments determined the effects of linking the selectable marker and the reporter gene on the same vector and the influence of transgene copy number. Finally, I describe an experiment demonstrating that the *Adh1* 5' MAR is in an open chromatin conformation *in vivo*.

In chapter V, I summarize the results of all of my experiments concerning the effects of MAR elements on transgene expression in maize cells and make some general conclusions about the epigenetic effects of flanking transgenes with MARs.

CHAPTER II. LITERATURE REVIEW

Matrix attachment regions (MARs) are DNA sequences that bind to the proteinaceous network inside the nucleus called the nuclear matrix. MAR elements are commonly found flanking the 5' and 3' ends of genes (Table 1), and are characteristic features of chromatin from eukaryotes as diverse as single-celled yeast to complex multicellular organisms, such as humans and maize (Amati and Gasser, 1988; Sykes et al., 1988; Avramova and Bennetzen, 1993). MARs are thought to be involved in maintaining chromatin in either an open active state or a compacted inactive state, and in insulating chromosomal domains from the regulatory elements of neighboring domains. Many models have been proposed for the action of DNA sequence elements that insulate genes from the effects of surrounding chromatin (Wolffe, 1994). In one class of models, the elements impair activation of a gene by blocking interaction of the promoter with enhancers or transcription factors outside of its domain. In the other class of models, the transcriptional activity is maintained by the element blocking the diffusion or assembly of repressive chromatin proteins.

Matrix attachment regions were originally divided into two groups of elements based on the way they were isolated. Those isolated by high salt extraction were called matrix attachment regions (MARs) and those isolated by lithium diiodosalicylate (LIS) were called scaffold attachment regions (SARs). Since it has been shown that both extraction methods give residual structures that bind the same DNA regions (e. g. Avramova and Bennetzen, 1993), many authors now consider MARs and SARs equivalent, and the MAR terminology is most frequently used. Throughout the rest of this thesis, DNA sequences

Table 1. Identified MAR Elements

Gene	Region	Organism	Reference
HSP70	5'	*Drosophila*	(Mirkovitch et al., 1984)
κ-immunoglobulin	J-C intron	mouse	(Cockerill and Garrard, 1986)
fushi-tarazu	5' & 3'	*Drosophila*	(Gasser and Laemmli, 1986a)
Sgs-4	5' & 3'	*Drosophila*	(Gasser and Laemmli, 1986a)
alcohol dehydrogenase	5' & 3'	*Drosophila*	(Gasser and Laemmli, 1986a)
ARS1	3'	yeast	(Amati and Gasser, 1988)
interferon-β	5' & 3'	human	(Bode and Maass, 1988)
β-globin	5'	murine	(Greenstein, 1988)
β-globin	5' & 3'	human	(Jarman and Higgs, 1988)
lysozyme	5' & 3'	chicken	(Phi-Van and Strätling, 1988)
HPRT	1st intron	human	(Sykes et al., 1988)
apolipoprotein B	5' & 3'	human	(Levy and Fortier, 1989)
α-globin	3'	chicken	(Farache et al., 1990)
rb7-5A	3'	tobacco	(Hall et al., 1991)
plastocyanin	3'	pea	(Slatter et al., 1991)
lectin	5' & 3'	soybean	(Breyne et al., 1992)
Adh1	5'	maize	(Avramova and Bennetzen, 1993)
Gmhsp 17.6-L	3'	soybean	(Schöffl et al., 1993)
ST-LS1	5' & 3'	potato	(Dietz et al., 1994)
Mha1	5'	maize	(Jin and Bennetzen, 1994, this work)
β-phaseolin	5' & 3'	*Phaseolus*	(van der Geest et al., 1994)
Adh1	3'	maize	(Avramova et al., 1995)
Heat Shock Cognate 80	5' & 3'	tomato	(Chinn and Comai, 1996)
plastocyanin	5' &3'	*Arabidopsis*	(van Drunen et al., 1997)

that associate with matrix or scaffold proteins *in vivo* and *in vitro* will be referred to as MARs.

2.1 The Nuclear Matrix

The network of proteins in the eukaryotic cell that form the nuclear matrix was first discovered following extraction of nuclei with high salt (Berezney and Coffey, 1974). The

residual structure defined as the matrix maintained the shape of the nucleus even after numerous chemical treatments. The matrix was composed of 98% protein, but also contained some DNA and RNA. In a subsequent report, Berezney's group found that DNA replication appeared to initiate within the nuclear matrix (Berezney and Coffey, 1975).

Proteins that have been associated with the nuclear matrix can be divided into three groups: structural proteins such as matrins (Belgrader et al., 1991), high-mobility group proteins (Zhao et al., 1993), and lamins (Luderus et al., 1994); regulatory proteins such as topoisomerase II (Cockerill and Garrard, 1986; Gasser et al., 1986), and replication machinery proteins (McCready et al., 1980); and proteins that bind unwound AT-rich DNA such as SATB1(Dickinson et al., 1992; Nakagomi et al., 1994; Liu et al., 1997), ARBP (for attachment region binding protein) (von Kries et al., 1991), and nucleolin (Dickinson and Kohwi-Shigematsu, 1995).

In addition to Berezney's early work on interphase nuclei, Laemmli's group identified a non-histone protein structure responsible for the basic shape of metaphase chromosomes (Paulson and Laemmli, 1977). When histones were removed from metaphase chromosomes *in vitro*, loops of DNA anchored to the proteinaceous matrix were observed. Eventually, Laemmli's group proposed that attachment sites in interphase chromosomes may also be the attachment sites they observed in metaphase chromosomes (Gasser and Laemmli, 1987). In a study published a short time later, they reported that MARs, previously identified as binding to interphase nuclear protein preparations, specifically bound metaphase protein preparations as well (Mirkovitch et al., 1988). The relationship between the matrix anchoring loops of chromatin in metaphase chromosomes and the nuclear

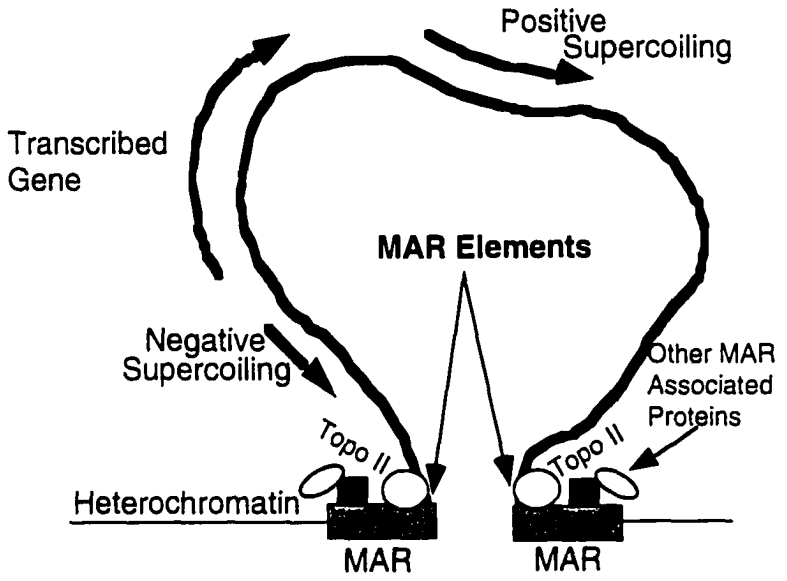


Figure 1. Diagram of a chromatin loop. Loop size can vary from 5 kp to greater than 100 kb. MAR elements bind to the proteinaceous nuclear matrix separating the active region from the surrounding heterochromatin.

matrix of interphase nuclei then became a hotly contested area of research, which is still not completely resolved to this day.

2.2 The Loop Model of Chromatin Structure

Shortly after the discovery of chromatin loops, a model for chromatin organization was proposed (Laemmli et al., 1978; Marsden and Laemmli, 1979). The model invokes specific chromatin attachment sites spaced at regular intervals that organize and separate chromatin into discrete functional loops (Figure 1). Genes within each loop are thought to be insulated from the regulatory effects of heterochromatin or transcriptional enhancers and silencers in neighboring loops. A prediction of this model is that transgenes flanked by MAR elements should also form a discrete loop upon integration, and function indepen-

dent of the surrounding chromatin. Thus, MAR-flanked transgenes should be expressed in a position-independent, copy-number-dependent fashion.

2.3 Identification and Isolation of Matrix Attachment Regions

The first MAR element described was a 657 bp fragment found in the cluster of Drosophila hsp70 heat-shock genes (Mirkovitch et al., 1984). This MAR was identified by developing a new milder method of extraction employing LIS that was less prone to the "sliding" of attachment points. Many more MAR elements have since been discovered from various organisms, including most recently, both monocotyledenous and dicotyledenous plants (Table 1). The ubiquitous nature of MAR elements as components of eukaryotic chromatin indicates they are likely to play an important and fundamental role in nuclear architecture.

Two basic methods are employed in the isolation of MAR elements. The first method determines whether a sequence is bound endogenously to the matrix, whereas the second method determines whether an exogenous DNA sequence can bind to an isolated nuclear matrix. The first method is useful for determining whether a sequence is really bound to the nuclear matrix *in vivo*. However, it is possible that sequences that are not initially bound to the matrix bind during the isolation process. Using the second method, many labs have shown that MAR sequences from one species can bind nuclear matrices from other species.

For each of the two MAR assays, the first three steps are identical. The first step is to remove the histones either with 2 M sodium chloride (Berezney and Coffey, 1974), or with

LIS (Mirkovitch et al., 1984). The second step is to digest away the DNA that is unassociated with the matrix using various restriction enzymes. The third step is to separate the matrices from the solublized DNA by centrifugation. The fourth step is where the two isolation methods differ. In the endogenous assay, the supernatant and the pellet fraction are run on a gel (in separate lanes), along with a lane containing both the pelleted nuclear matrix and the solubilized DNA. The gel is then blotted and probed with labeled putative MAR fragments. The fragments that hybridize in the pellet lane and the combined lane are MARs. In the exogenous assay, the pelleted matrices are incubated with end-labeled fragments and competitor DNA (frequently sheared *E. coli* DNA). The mixture is then separated by centrifugation, run on a gel, and blotted. Bands that hybridize in the pellet lane are MAR elements.

2.4 Properties of MAR Elements

2.4.1 Physical properties

MAR elements are typically 300-2000 bp in length and greater than 70 percent AT-rich. By attaching to the nuclear matrix, they create loops of chromatin 5-200 kb long. Most MARs also contain many conserved AT-rich motifs (Laemmli et al., 1992). One of these motifs is the consensus topoisomerase II cleavage site (GTNWAYATTNAT-NNR)(Gasser and Laemmli, 1986b; Gasser et al., 1986). When fragments containing this motif are concatemerized, they form a MAR with artificially high binding affinity for the nuclear matrix (von Kries et al., 1991; Girard et al., 1998). Although MARs often show poor sequence conservation, MAR binding is generally conserved across species (Mielke

et al., 1990; von Kries et al., 1991; Breyne et al., 1992). MAR sequences often have a high capacity for unwinding *in vivo* and may exist as partially single stranded regions (Bode et al., 1992; Dickinson et al., 1992; Boulikas, 1995). Recently, Singh et al. (1997) wrote a computer program that uses these physical properties to identify putative MAR elements in DNA sequences.

2.4.2 Genetic properties

Four genetic properties of MARs have been summarized in a recent review (Holmes-Davis and Comai, 1998). First, some MARs act as boundary elements by functionally insulating the chromatin they flank from the influences of surrounding chromatin. As boundary elements, MARs prevent the spread of heterochromatin and insulate genes from the effects of transcriptional enhancers or silencers in neighboring loops (Gasser and Laemmli, 1987; Geyer, 1997). Second, MAR elements can play a role in regulation of chromatin structure and affect gene expression more directly, e. g. by binding regulatory *trans*-acting factors or by disrupting proteins involved in chromatin condensation such as histone H1. Third, MARs may function as origins of replication (Berezney and Coffey, 1975; Gasser, 1991). For example, some yeast origins of replication (including ARS1) have been identified as MAR elements and/or transcriptional silencers (Amati and Gasser, 1988). The final role proposed for MAR elements is in mitotic chromosome organization, where they may be involved in chromosome folding and chromatin compaction (Marsden and Laemmli, 1979).

2.5 MAR Effects on Transgene Expression in Animal Systems

The first indication that MARs might have an effect on transgene expression came from studies using the β-globin locus control region (LCR)(Grosveld et al., 1987). When the human β-globin gene was flanked with sequences that included the LCR and introduced into transgenic mice, each transgenic animal expressed the human β-globin transgene at levels equal to that of the endogenous mouse gene, regardless of the insertion site. However, the LCR was known to contain transcription factor binding sites and other sequences critical for β-globin gene regulation. In a later study, it was shown that the LCR regions that Grosveld et al. had used in their study included MAR elements (Jarman and Higgs, 1988). Flanking a neo transgene with the MAR element located at the 5' end of the β-globin gene conferred position-independent expression, when the polyoma virus enhancer was included in the transgene constructs (Yu et al., 1994). Interestingly, neither the polyoma virus enhancer nor the β-globin 5' MAR alone could reduce position effects (Yu et al., 1994).

The first experiment specifically testing the effects of MARs on transgene expression used A-elements derived from flanking regions of the chicken lysozyme gene (Stief et al., 1989). The 5' A-element increased transgene expression approximately 10-fold, independent of its chromosomal position, when transformed into chicken promacrophages. Later, Stief et al. demonstrated that the chicken lysozyme MAR could also reduce position effect variation in transgene expression in a cell line of another species, specifically rat fibroblasts (Phi-Van et al., 1990). In another study, the mouse whey acidic protein was flanked with the chicken lysozyme MAR, and 11 out of 11 lines with MARs expressed the gene,

compared to 50% of the lines generated using transgenes without flanking A-elements (McKnight et al., 1992). In addition, 4 out of 5 of the lines analyzed showed proper regulation of the gene. Finally, Klehr et al. (1991) showed that flanking transgenes with MAR elements from either animals or plants increased the average expression level of transformants in mouse cell lines, indicating that MARs could function across very wide species boundaries.

Cell lines containing MAR flanked transgenes have also been found to exhibit higher levels of gene expression in the presence of sodium butyrate than cell lines containing transgenes without MARs (Klehr et al., 1992). Butyrate is a short fatty acid that can stimulate gene expression by inhibiting histone deacetylases. Histone acetylation is associated with open chromatin and access to the transcription machinery. Since the enzymes involved in histone acetylation and deacetylation are thought to be components of the nuclear matrix (Hay and Candido, 1983; Hendzel et al., 1991), it is possible that flanking MAR elements can facilitate histone acetylation in the presence of butyrate (Klehr et al., 1992).

2.6 MAR Effects on Transgenes in Plant Systems

Results of these early animal studies generally showed that MARs increase the average expression level of transgenes and decrease the variation in expression from transformant to transformant, although there are many inconsistencies apparent in different studies. These features are consistent with MARs having a boundary element function. However, results from the first experiments performed in plants varied considerably from

these early animal studies. In the first study where MAR elements were tested in plant cells, Breyne et al. (1992) found that flanking a transgene with a soybean MAR element decreased the average expression 2-fold, and that this also decreased the variability in expression. A human MAR had no effect on transgene expression in their tobacco cell lines. In contrast, Allen et al. (1993) found that a MAR from yeast that bound tobacco nuclear proteins could increase the average transgene expression level 24-fold. In another study using tobacco plants, Mlynarova et al. (1994) reported that flanking transgenes with the chicken lysozyme MAR element reduced position effects and resulted in copy number dependent transgene expression. The conclusions from each of these studies illustrate the variability of effects that MARs can have on transgene expression.

More recent work done with MARs in plants has indicated that they generally can increase the average expression level among transformants, but relatively few studies have reported significantly decreased variation of transgene expression levels or copy number dependent expression (Table 2).

2.7 Gene Silencing

Most transgenic studies using MARs were done in the hopes of preventing gene silencing. Silencing has been studied extensively in both animals and plants. In plants, much of the focus has been primarily on homology dependent silencing of transgene (Matzke and Matzke, 1995; Jorgensen et al., 1996). Homology dependent silencing phenomena can be classified into one of two types; transcriptional or post-transcriptional. Transcriptional silencing generally involves either multiple linked copies of a transgene at

Table 2. Summary of transgenic studies using MAR elements (Holmes-Davis and Comai, 1998)

Source of MAR	Transform-ants per construct	Cell type or organism	Copy number	Increase in level of expression	Copy number dependent expression	Reduction of position effects	Reference
human β-globin	40	mice	1 to >100	none	yes	yes	(Grosveld et al., 1987)
chicken lysozyme	10-19	chicken pro-macrophages	1-90	10 fold	yes	yes	(Stief et al., 1989)
human *interferon-β*		mouse L cells		20-30 fold			(Klehr et al., 1991)
chicken lysozyme	12-14	rat-2 fibroblasts	1-270	4.5 fold	sort of	"dampened"	(Phi-Van et al., 1990)
mouse κ-immunoglobulin		mouse plama-cytoma cells	1	4 fold	N/A	no	(Blasquez et al., 1989)
Drosophila hsp70	16-27	HeLa		20-40 fold	no	no	(Poljak et al., 1994)
human apolipoprotein B		human and rat hepatoma cells	1	200-fold	N/A	yes	(Kalos and Fournier, 1995)
soybean lectin	~60	tobacco calli	N/D	- 2 fold	N/D	yes	(Breyne et al., 1992)
human β-globin	~60	tobacco calli	N/D	slight	N/D	none	(Breyne et al., 1992)
yeast ARS1	~30	tobacco calli	18-160	24-fold	no	slight	(Allen et al., 1993)
soybean Gmhsp 17.6-L	8-18	tobacco plants	N/D	7-9 fold	N/D	no	(Schöffl et al., 1993)
chicken lysozyme	46-60	tobacco plants	1 to >3	3-7 fold	no	yes	(Mlynarova et al., 1994)
β-phaseolin	4-20	tobacco plants	1-2	1.5 fold	yes	yes	(van der Geest et al., 1994)
chicken lysozyme	>60	tobacco plants	1 to >3	2 fold	no	yes	(Mlynarova et al., 1995)
tobacco *rb7-5A*	16-17	tobacco calli	1-31	140-fold	no	no	(Allen et al., 1996)
tomato *HSC80*	11-32	tomato plants	1-5	yes	no	unclear	(Chinn and Comai, 1996)
Phaseolus β-phaseolin	2-10	tobacco plants	N/D	yes	N/D	no	(van der Geest and Hall, 1997)

a single site, or alternatively, a transgene and a homologous "target" gene located elsewhere in the genome (Matzke and Matzke, 1995). With this form of silencing, no transcripts are initiated from any of the silenced gene copies. When one of the transgenes becomes silenced, which usually correlates with an increase in methylation of the gene, this copy can then act as a silencer for other copies of the gene, either in *cis*- or in *trans*-. This may be a system for silencing endogenous transposable elements (Henikoff and Matzke, 1997)

Post-transcriptional homology dependent silencing (also called cosuppression (Napoli et al., 1990)) may be caused by a threshold mechanism which senses the level of homologous mRNAs in the cytoplasm and activates a process for silencing when that threshold is reached (Smith et al., 1994). Post-transcriptional silencing is distinguished from other forms of silencing by the presence and continuous re-initiation of transcripts corresponding to silenced gene copies. Methylation may also be involved in this type of silencing (English et al., 1996), and it is generally thought that post-transcriptional silencing is a plant's defense mechanism for preventing expression and replication of RNA viruses (Baulcombe and English, 1996). Because most viruses cannot be transmitted through the germline, whereas transposons can, this may be why genes silenced by transcriptional mechanisms generally stay silent when transmitted through meiosis, whereas post-transcriptionally silenced genes regain full activity following meiotic segregation of the transgene responsible for silencing (Henikoff and Matzke, 1997).

Silencing has also been extensively studied in Drosophila and yeast. In Drosophila, two boundary elements have been identified: the suppressor of Hairy wing (su[Hw])-bind-

ing region found in gypsy retroelements and specialized chromatin sequences (scs/scs') found flanking the 87A7 *hsp70* locus (Geyer, 1997). These elements can prevent the spread of heterochromatin and block enhancer function when placed between the enhancer and promoter (Holdridge and Dorsett, 1991; Geyer and Corces, 1992; Kellum and Schedl, 1992; Roseman et al., 1993; Galliano et al., 1995). Several proteins have been identified that are necessary for the function of these elements. For the su(Hw)-binding region, the su(Hw) and mod(mdg4) proteins are required and for the scs elements, boundary element associated factor (BEAF) protein is required (Geyer, 1997).

A mosaic type of gene silencing called position-effect variegation (PEV) has been studied using the eye color genes in Drosophila. This type of silencing is seen when an endogenous gene is placed near a region of heterochromatin through chromosomal rearrangement or when a transgene integrates within a region of heterochromatin. Henikoff (1996) has suggested a pairing-looping model to account for PEV silencing. In this model, pairing of a gene in a euchromatic region and a homologous gene in a heterochromatic region draws the euchromatic gene away from factors required for expression.

Studies in yeast have identified three stages of gene silencing; establishment, maintenance, and heritability (Loo and Rine, 1995). For establishment of silencing, the yeast cell must pass through S phase (Miller and Nasmyth, 1984; Lustig, 1998). The factors involved in establishment of silencing during passage though S phase have not yet been identified, although candidates include the origin recognition complex (ORC) and chromatin assembly factor-1 (CAF-1) (Lustig, 1998). Maintenance and heritability of gene silencing were distinguished by the elimination of the *HML* silencer using site-specific

recombination *in vivo* (Holmes and Broach, 1996). After elimination of the silencer, the *HML* locus remained silent until the next round of cell division. However, after cell replication, repression of the *HML* locus was gradually lost. This indicated that the *HML* silencer was not required for the maintenance of silencing within the cell cycle, but was required for heritability of the repressed state.

There are primarily three chromosomal regions where transcriptional silencing occurs in yeast; the telomeres and the *HMR* and *HML* loci. Although some of the factors involved in silencing at these regions differ, they all require the silent information regulators (Sir2p, Sir3p, and Sir4p) and histones H3 and H4 (Lustig, 1998). Recently, studies have found that SIR proteins also function in recombination and aging. Using two-hybrid analysis, Tsukamoto et al. (1997) found that Sir4p could interact with yeast Hdf1p. Hdf1p is the yeast homolog of a subunit of Ku, the mammalian protein complex involved with non-homologous DNA double strand break repair (Tsukamoto et al., 1997). Mutational analysis revealed that *SIR2*, *SIR3* and *SIR4* were also involved in end-joining in yeast. There is also a link between SIR proteins and aging: loss of *sir2*, *sir3*, or *sir4* function decreases the life span of the yeast cells, whereas a gain-of-function mutation (*SIR4-42*) increases life-span (Kennedy et al., 1997). This group also discovered that *SGS1*, the yeast homolog of the gene that causes Werner's syndrome (a human disease that results in premature aging) is involved (Sinclair et al., 1997). Mutations at *SGS1* cause relocalization of Sir3p, which results in loss of telomeric gene silencing and decreases the lifespan of the cells.

When I embarked on my dissertation project, I wished to address whether the inconsistencies in transgenic studies using MARs (Table 2) could be resolved with a more system-

atic study using larger sample numbers. Furthermore, I wanted to know if the inconsistency of MAR effects on transgene expression might be related to epigenetic phenomena. My results clearly demonstrate the epigenetic nature of MAR effects on transgene expression and parallel many of the observations linking silencing, end-joining and aging in yeast. Thus, I propose that the influence of MARs on transgene expression in maize cells may involve silencing proteins analogous to those encoded by the yeast *SIR* genes.

CHAPTER III. MATERIALS AND METHODS

3.1 Vector Construction

Plasmid maps for all vectors used in this work are shown in Figure 2. The plasmids PHP264 (Figure 2A), PHP1528 (Figure 2B), PHP3528 (Figure 2C), PHP5438 (Figure 2D), PHP5456 (Figure 2E), and PHP5459 (Figure 2F) were constructed at Pioneer Hi-Bred International, Inc. before I started this work. PHP264 consists of an enhanced CaMV35S promoter (bases -421 to -90 and -421 to +2, (Gardner et al., 1981)), a 79 bp fragment from the 5' leader sequence of tobacco mosaic virus (Gallie et al., 1987), the first intron of the maize *Adh1*-S gene (Dennis et al., 1984), the coding sequence of the *GUS* (β-glucuronidase) gene (Jefferson et al., 1987), and the potato proteinase II (*pinII*) gene (bases 2 to 310, (An et al., 1989)). PHP264 and all other plasmids in this work have pUC derived backbones. The vectors PHP1528 and PHP3528 are similar to PHP264 except for the coding sequence. PHP1528 contains the firefly luciferase gene (de Wet et al., 1987) and PHP3528 contains the *BAR* gene from *Streptomyces hygroscopicus* (Thompson et al., 1987). A 839 bp *Eco*RI to *Hind*III fragment region of ARS1 (Struhl et al., 1979) was modified via site-specific mutagenesis (Sambrook et al., 1989), so it contained a *Bam*HI site on the 3' end and a *Not*I site on the 5' end. This modified ARS1 fragment was then inserted into PHP1528 at the 5' and 3' ends of the 35S::luciferase cassette respectively, using standard cloning procedures (Sambrook et al., 1989), to create PHP5438 (Figure 2D). PHP5456 (Figure 2E) was made by replacing the luciferase coding sequence with the *BAR* coding sequence, using unique sites in the promoter and terminator. The 5' region of the maize *Adh1* gene (Dennis et al., 1984) was supplied in a pUC vector by our

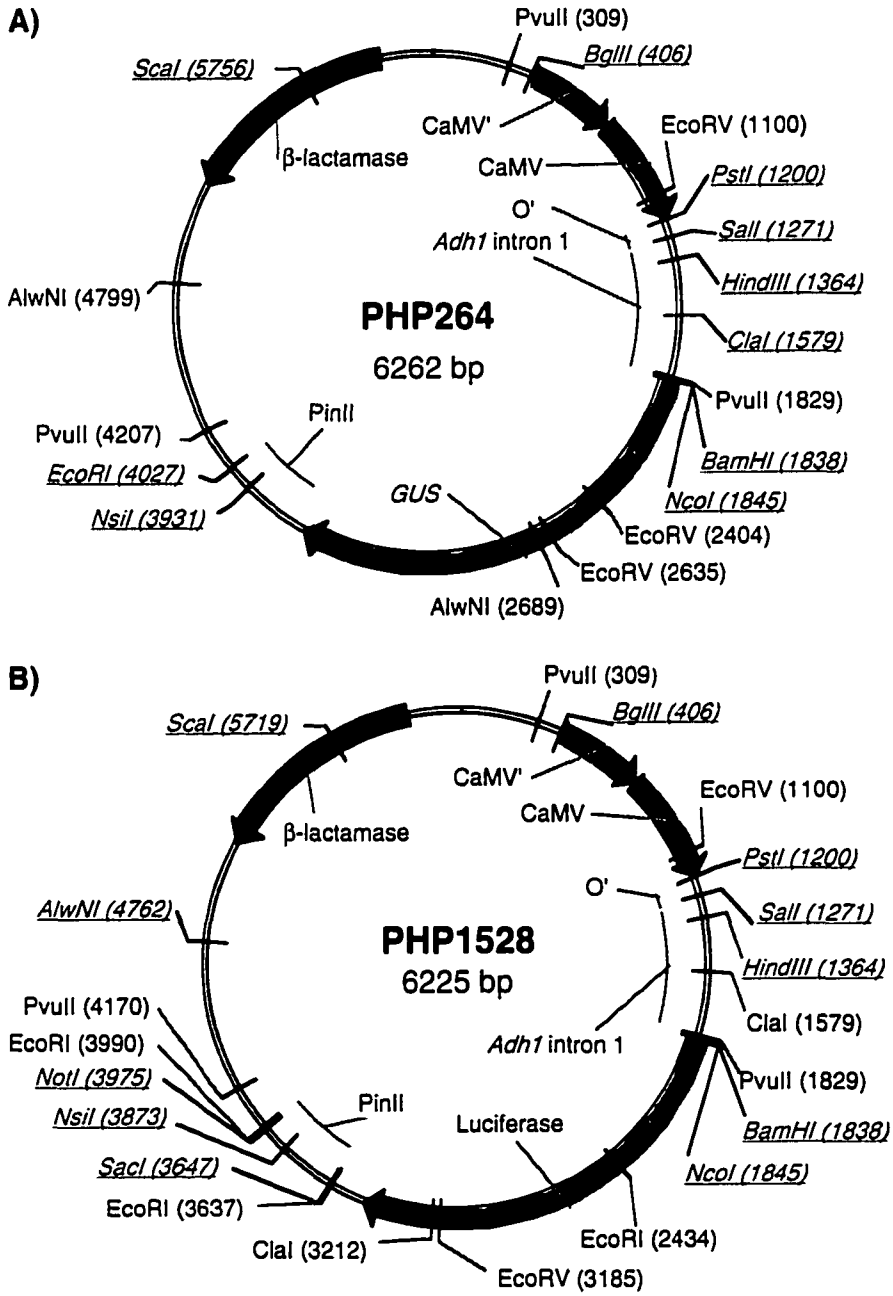
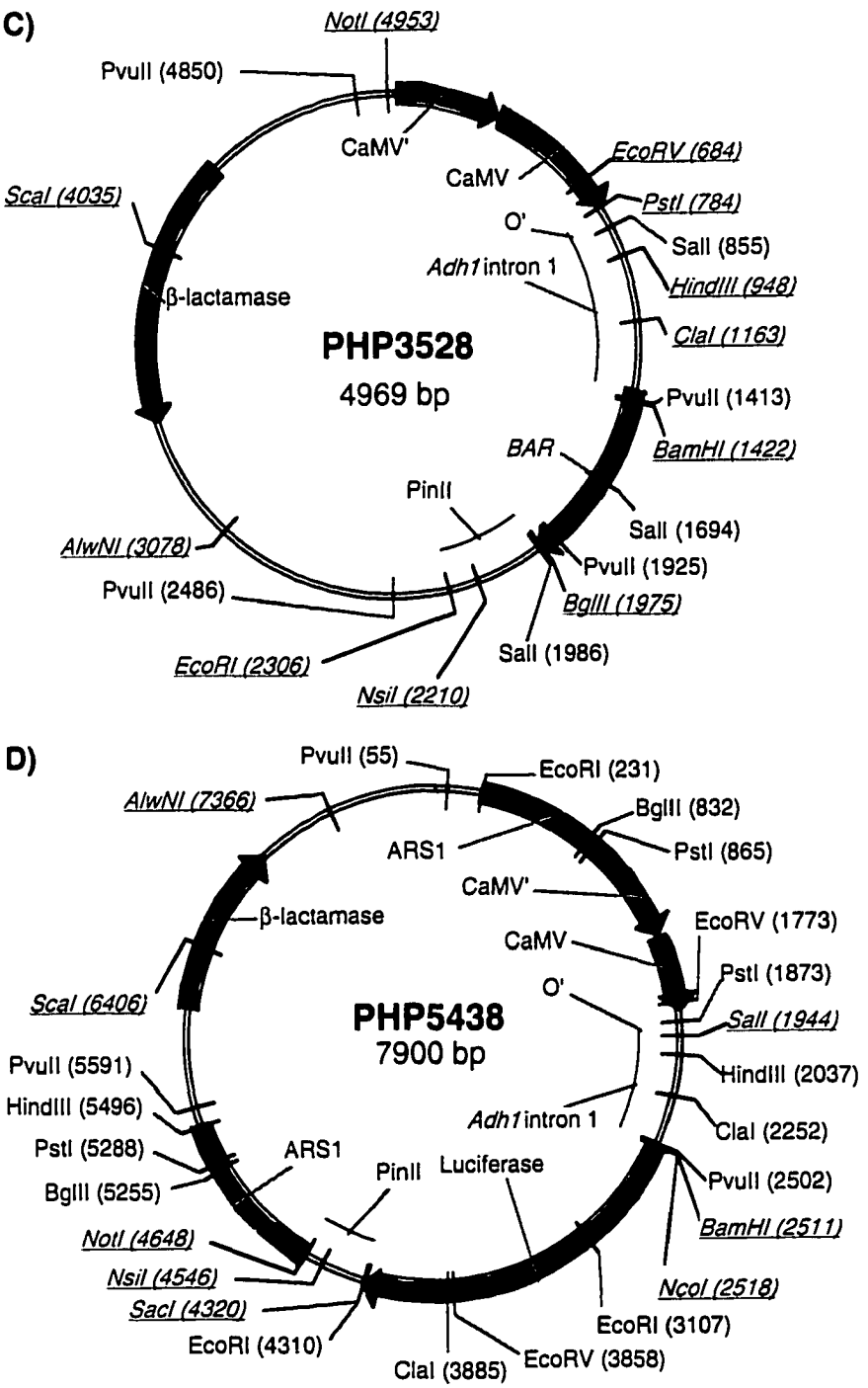


Figure 2. Plasmids used in this study. A-W are plasmids that were used in this study. Planning of plasmid construction was done on Gene Construction Kit (Textco) and VectorNTI (Informax, Inc.) for the Macintosh. Graphics were created by VectorNTI.



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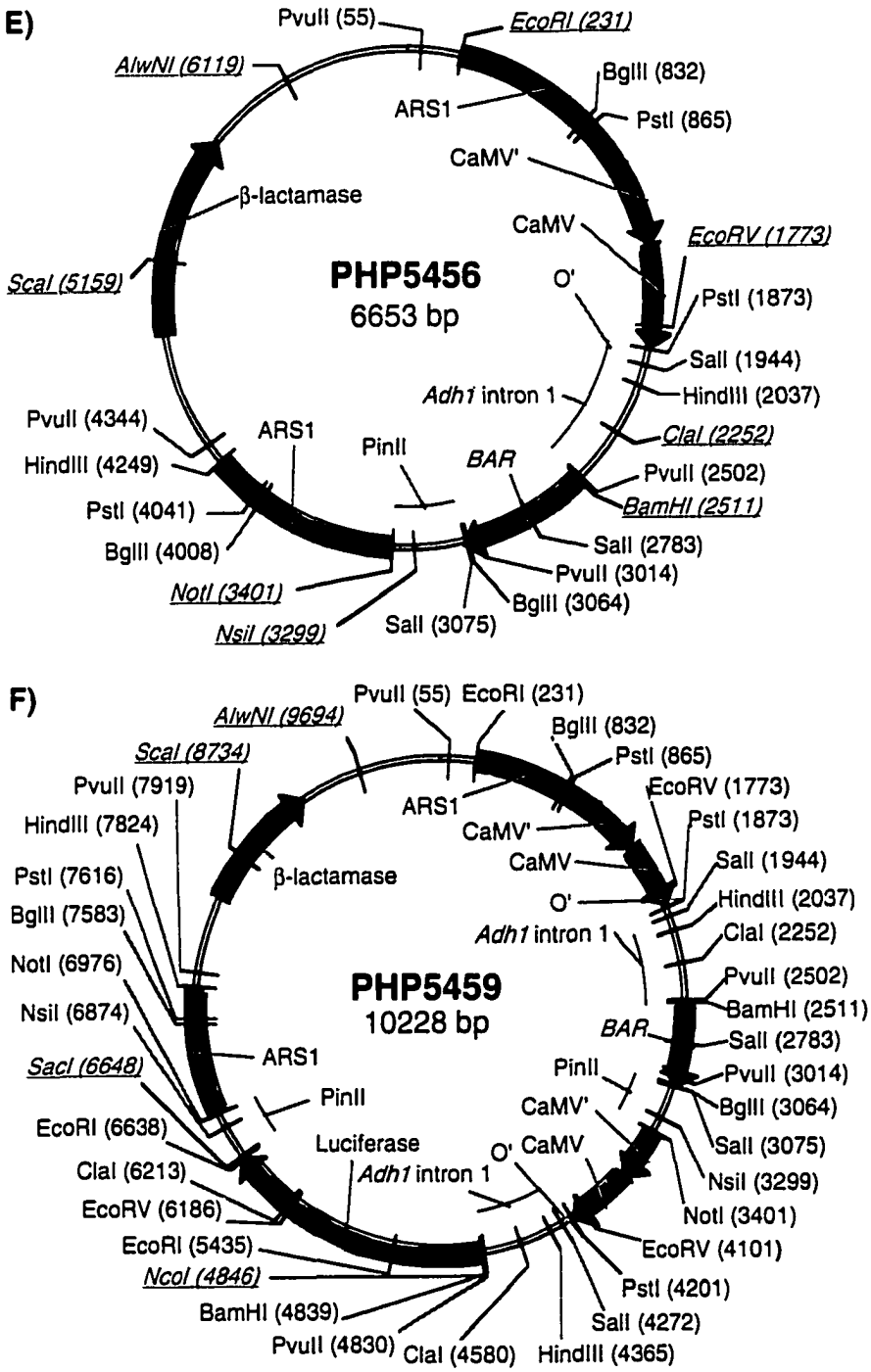

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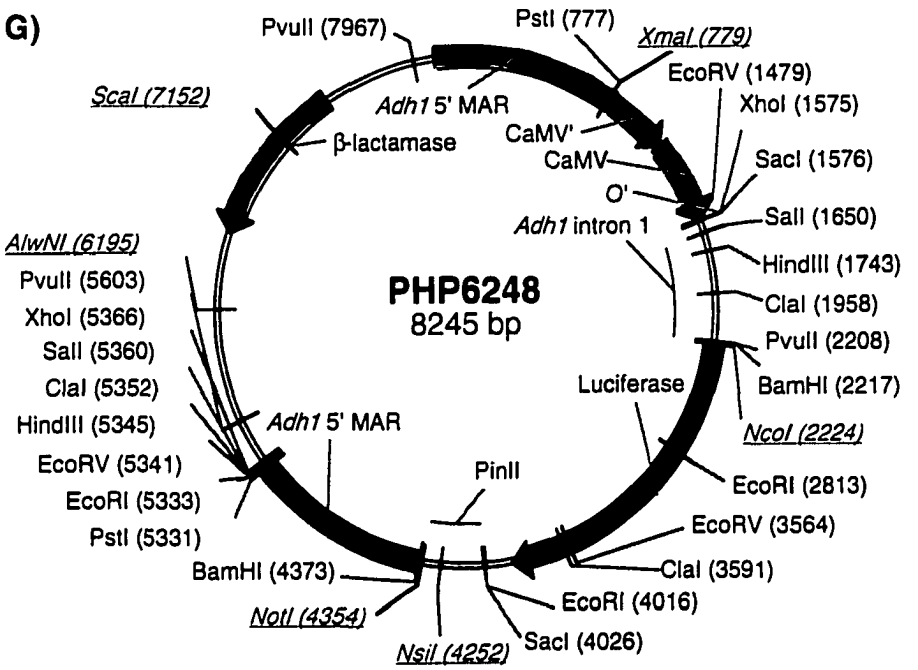

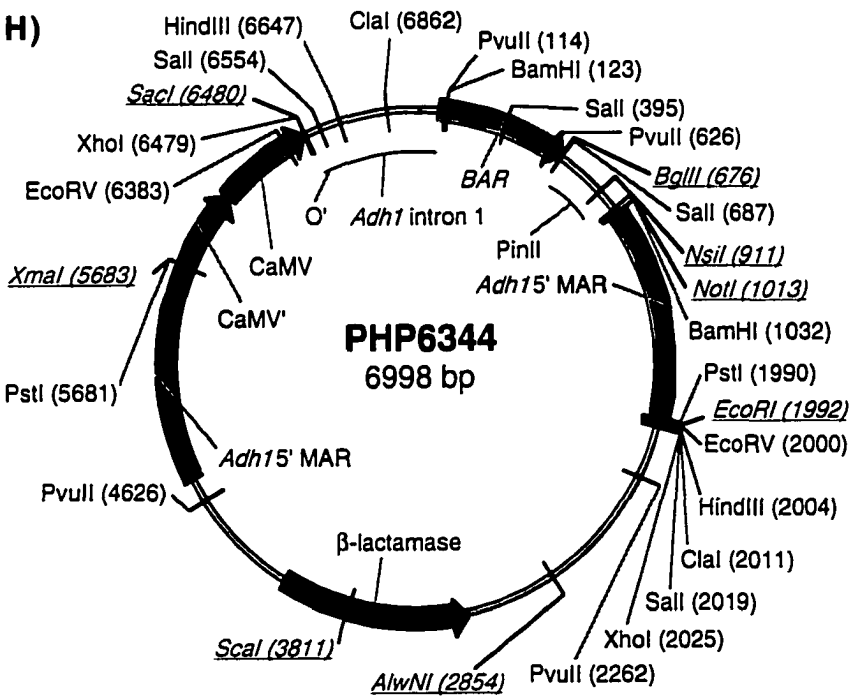

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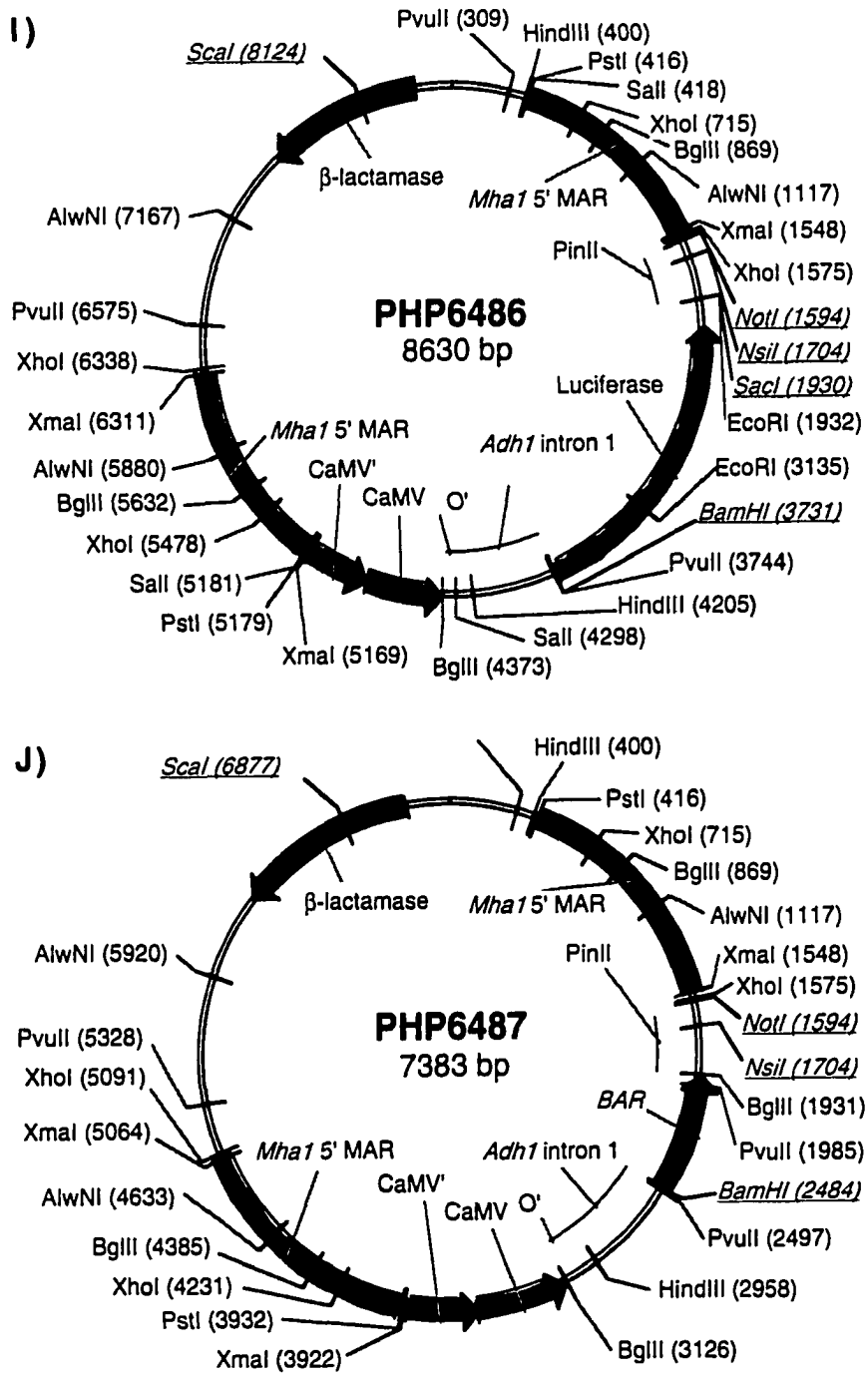

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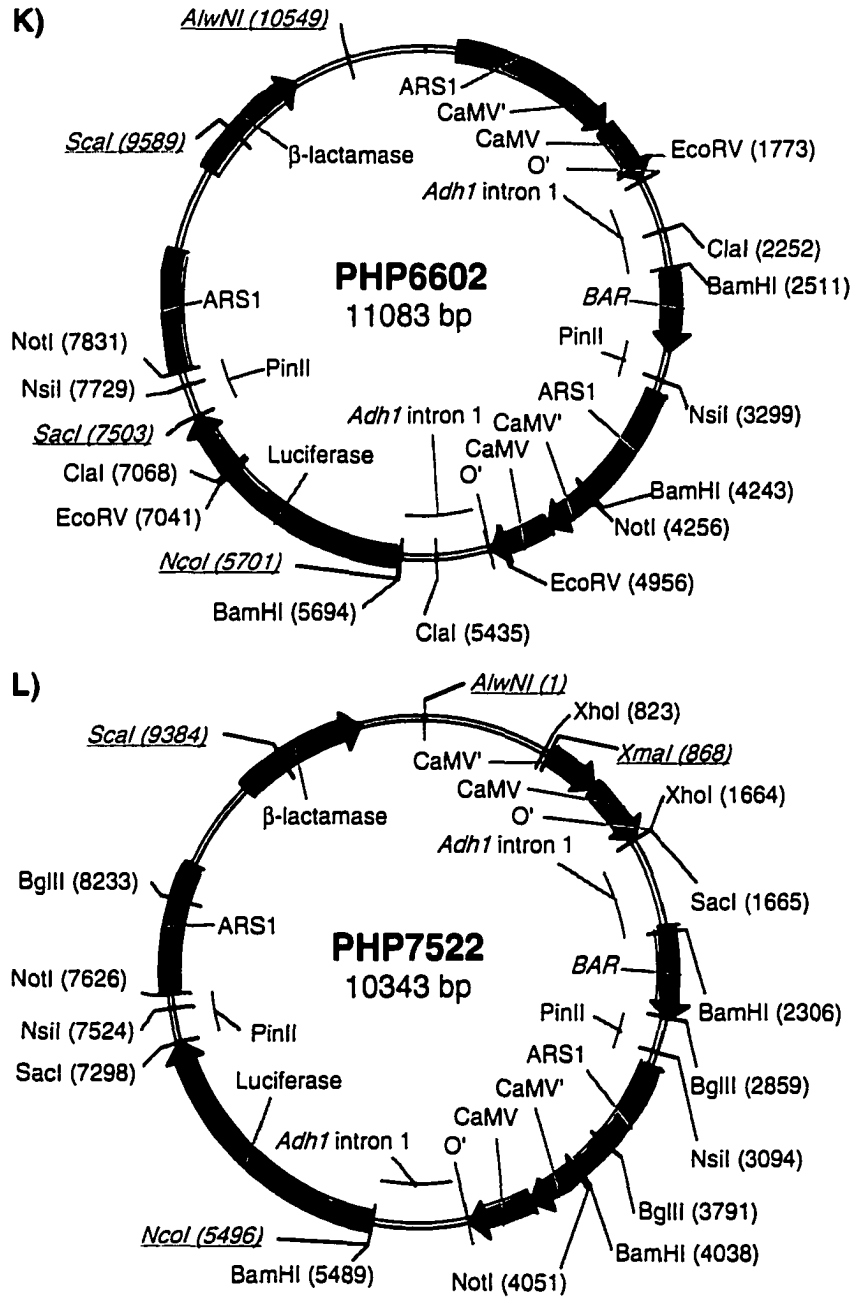

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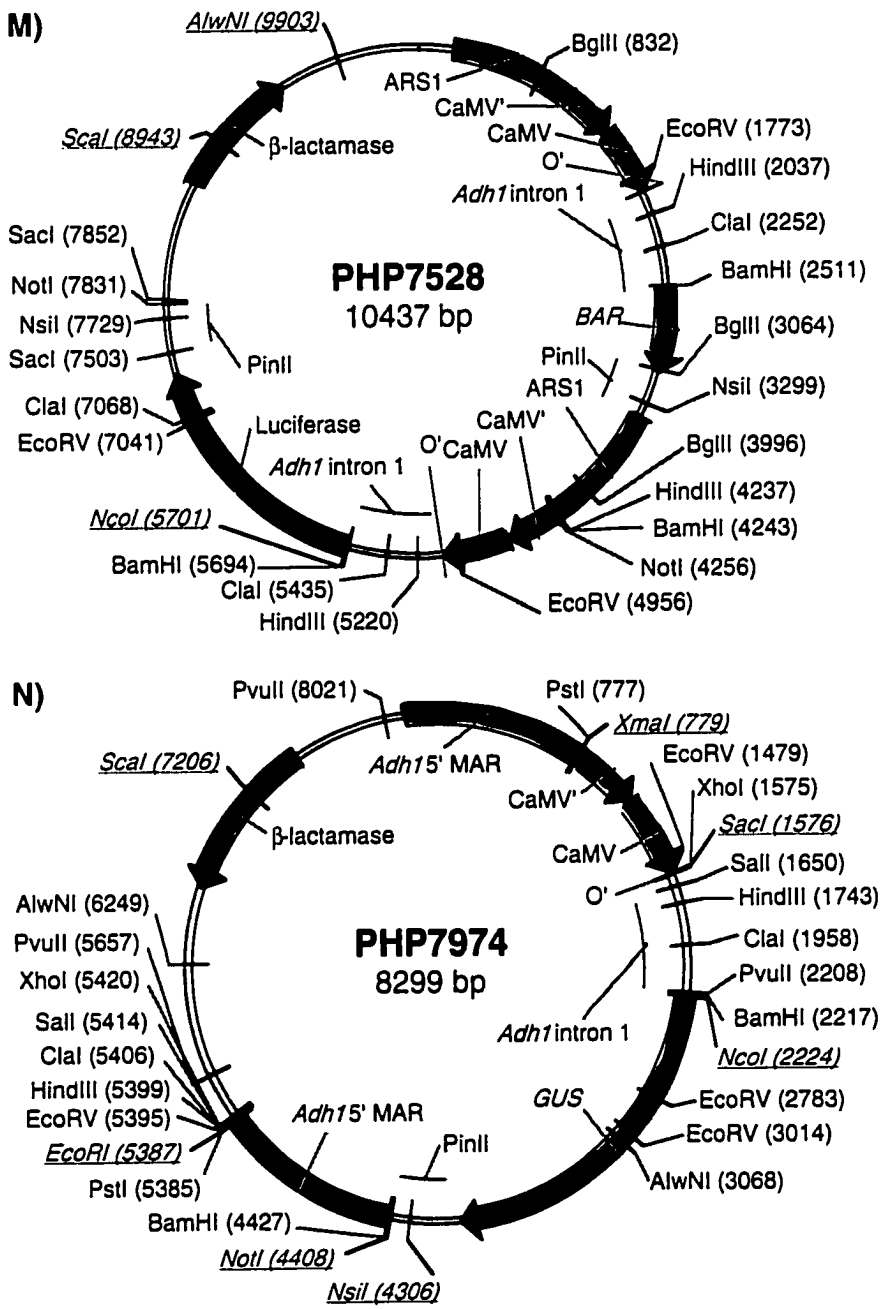

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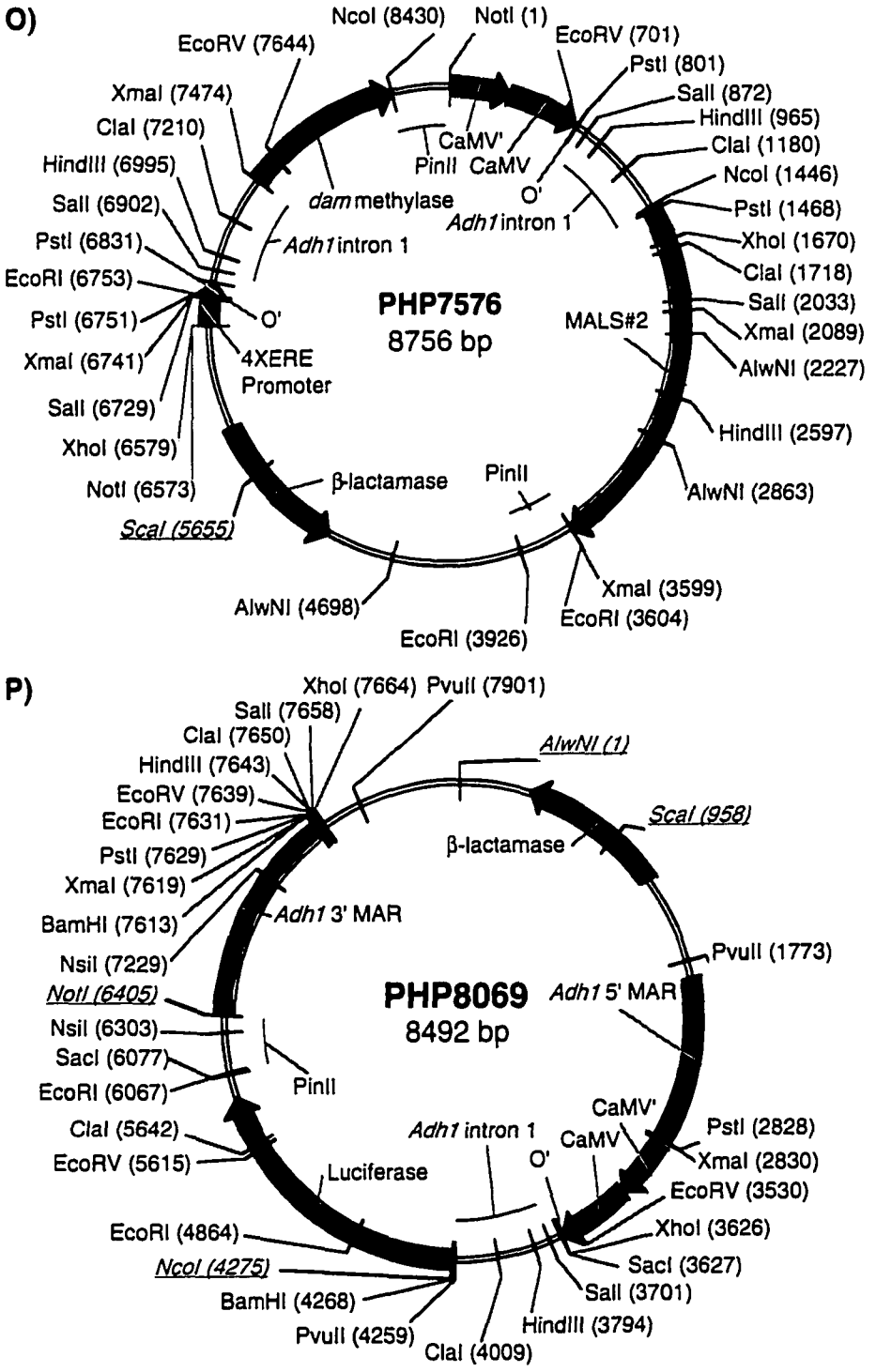

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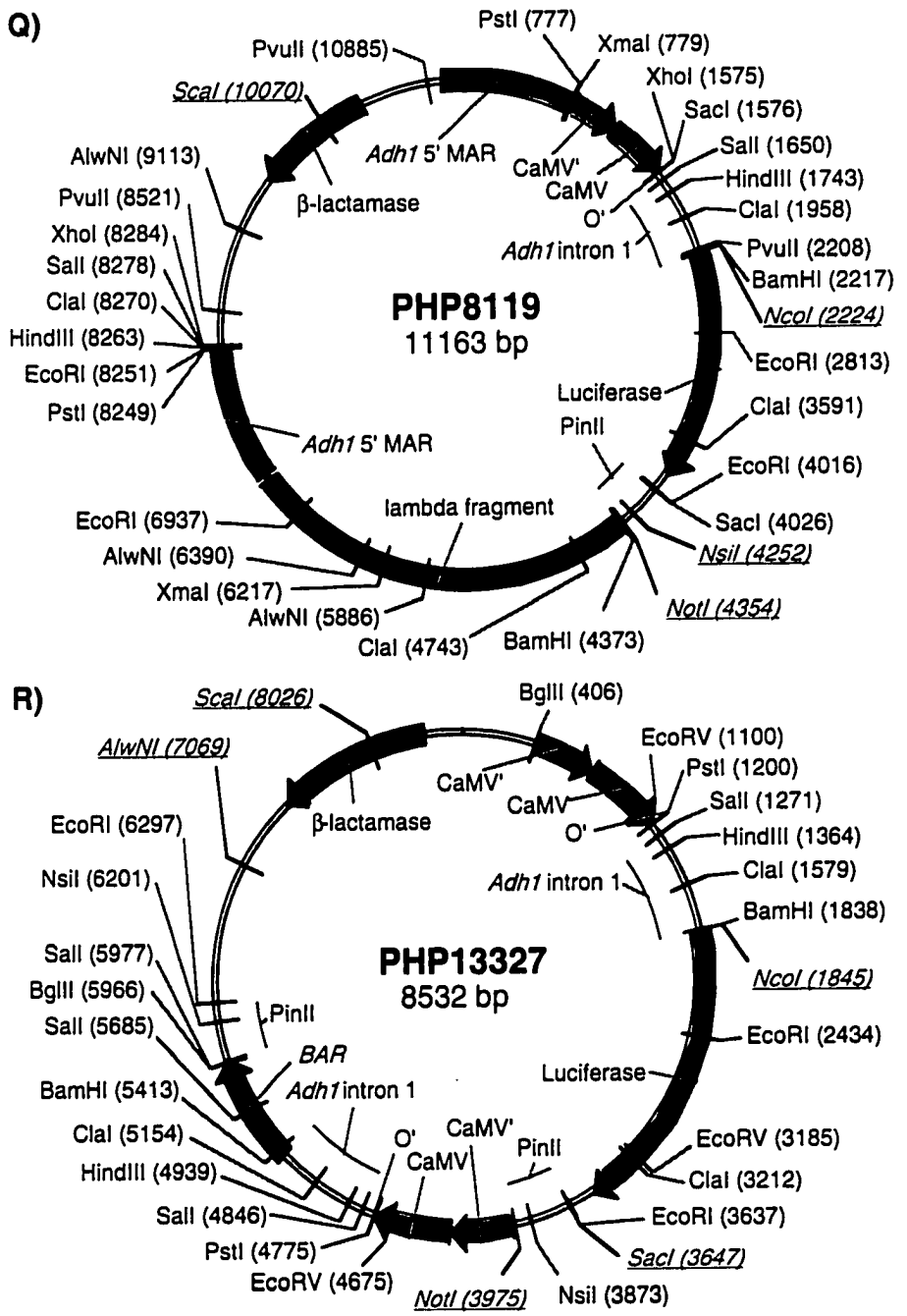

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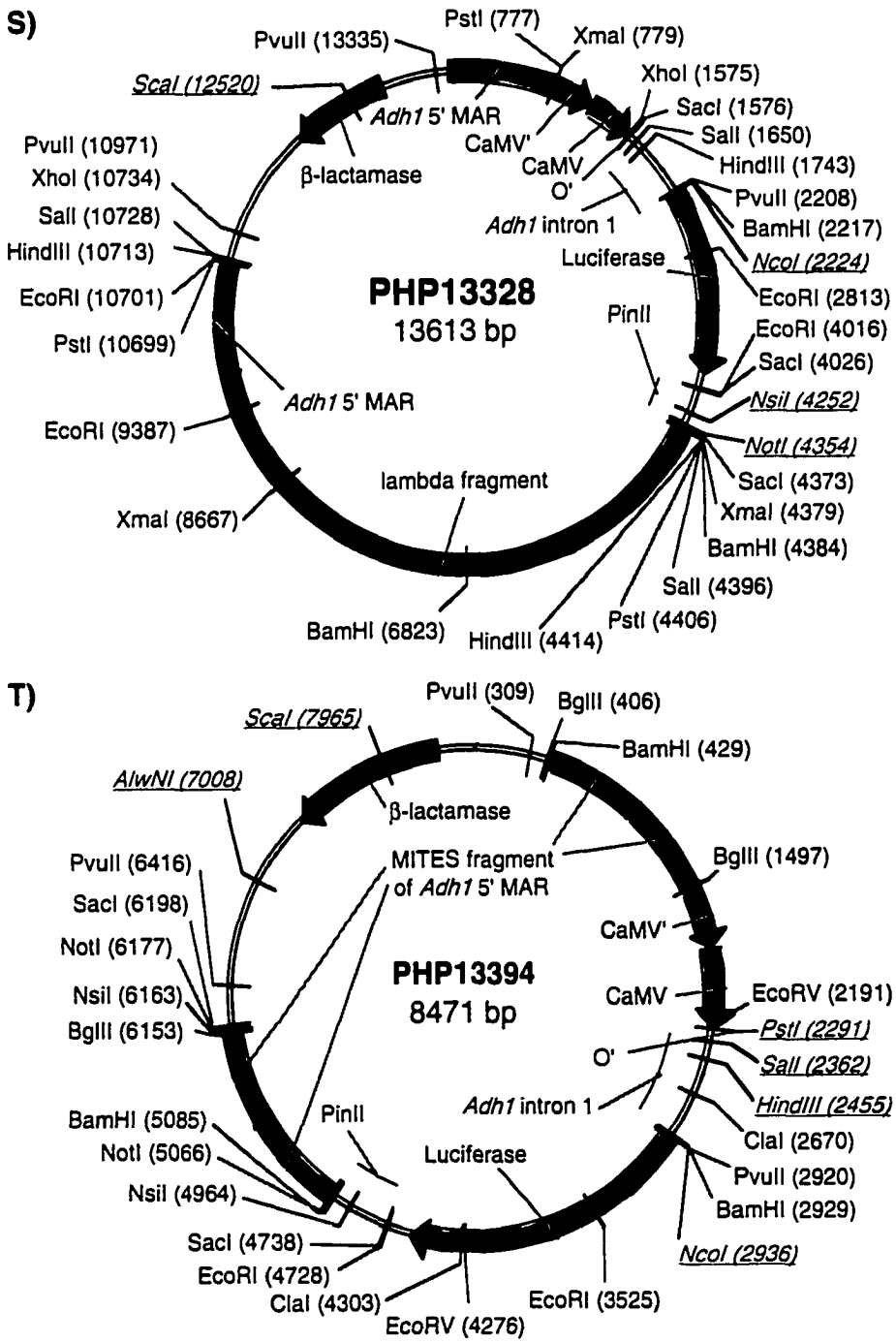

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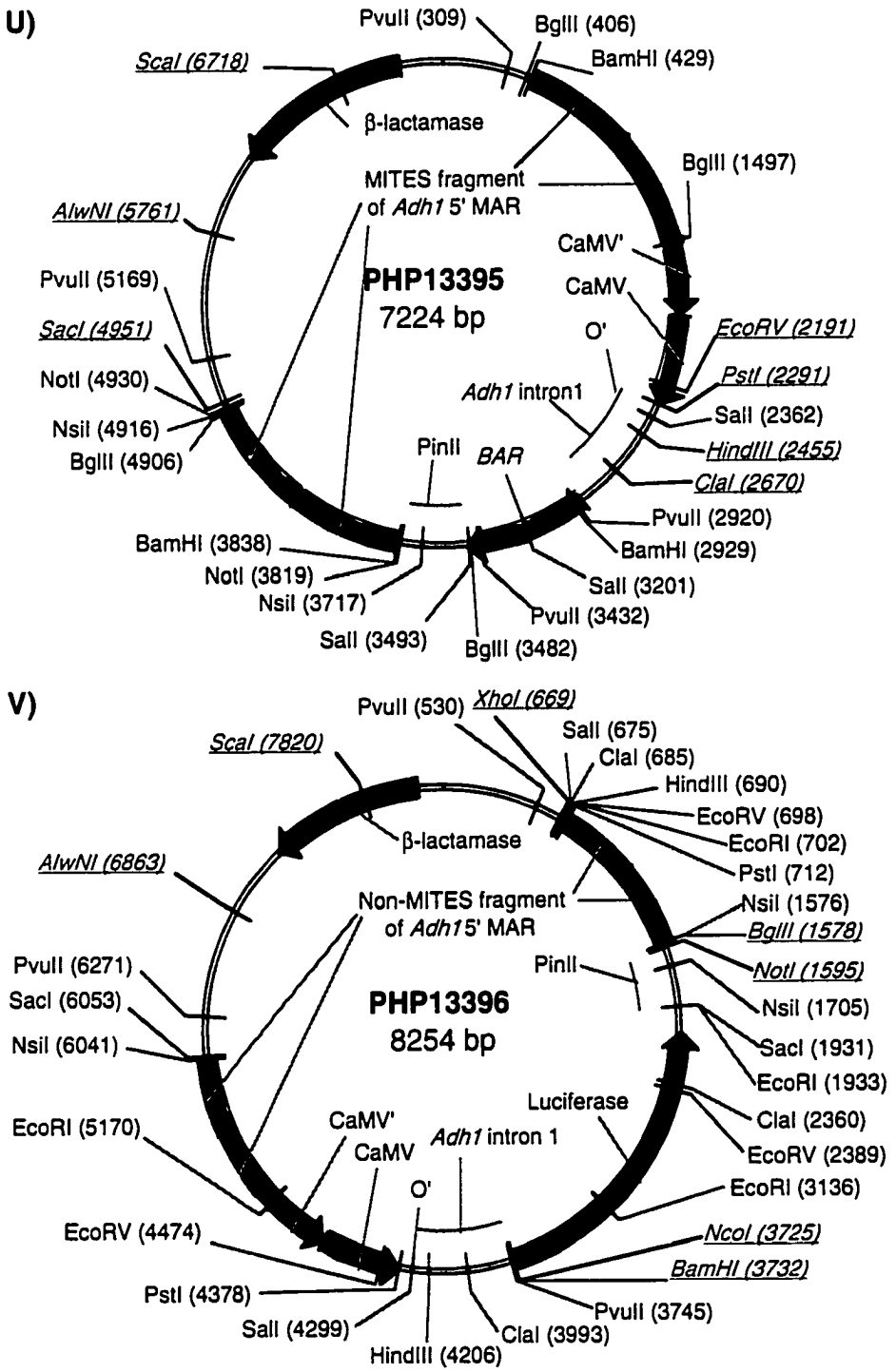

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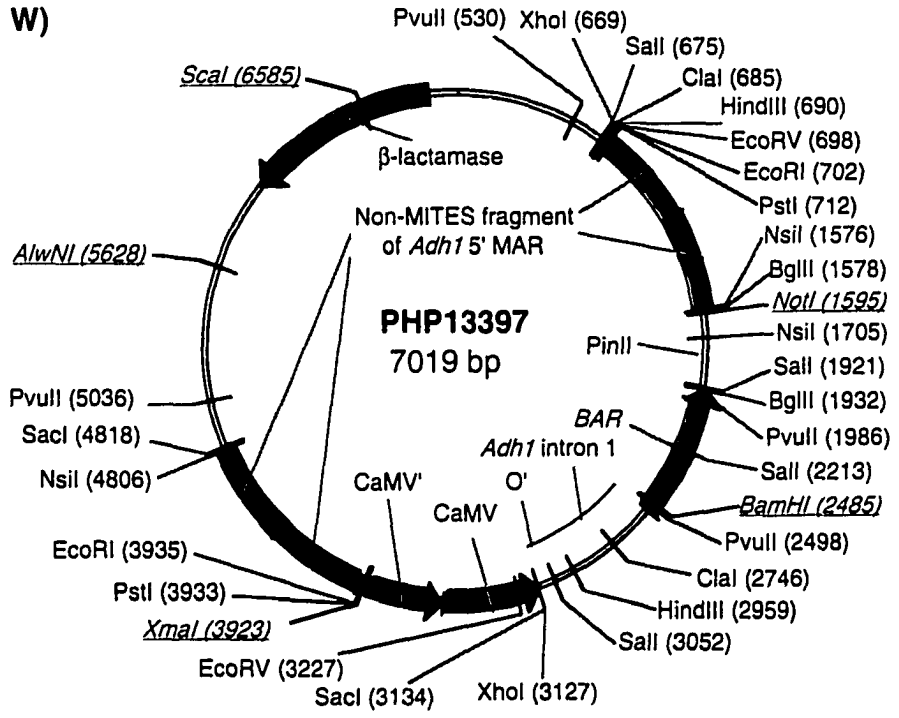


Figure 2. continued

collaborators Zoya Avramova and Jeff Bennetzen at Purdue University. The 948 bp *Bam*HI to *Pst*I fragment containing the *Adh1* 5' MAR (Avramova and Bennetzen, 1993) was cloned into Bluescript SK+ (Stratagene). The *Mha1* 5' MAR (-2562 to -1442 of Genbank entry U09989; (Jin and Bennetzen, 1994)) was also subcloned into pUC19. Vectors containing these MARs flanking luciferase or *BAR* (PHP6248, PHP6344, PHP6486, and PHP6487) (Figure 2G-J) were constructed by inserting the MARs into sites at the 5' or 3' end of PHP1528 or PHP3528, as described for PHP5438 and PHP5456. PHP7974 was constructed by removing the luciferase coding region from PHP6248, using unique sites in the *Adh1* intron and pinII terminator, and replacing it with the *GUS* gene from PHP264.

PHP8069 was constructed by replacing the downstream MAR with the *Adh1* 3' MAR, identified as a 1189 bp *Xba*I fragment located 8 kb downstream of the *Adh1* gene by our collaborators Zoya Avramova and Jeff Bennetzen. PHP8119 was constructed by inserting a 2.9 kb *Bgl*II to *Bam*HI fragment from phage lambda (38815-41738) between the pinII terminator and the MAR. PHP13328 was constructed by inserting a 5.3 kb *Bgl*II to *Hin-dI*II fragment from phage lambda (38815-44141) between the *pinII* terminator and the MAR. PHP7576 was constructed by replacing the luciferase coding sequence of PHP5947 (Roth et al., in prep.) with a 936 bp fragment of *dam* methylase (195-1131 of Genbank entry V00272; (Brooks et al., 1983)) and linking it in *cis*- to PHP1528 with the luciferase sequence replaced by a 1921 bp fragment of a mutant *Z. mays* acetohydroxyacid synthase coding sequence (605-2526 of Genbank entry X63554; (Fang et al., 1992)), which confers resistance to the herbicide chlorosulfuron (Fromm et al., 1990).

Two copies of the region from -1201 to -680 of *Adh1* containing MITEs (Figure 31) were placed end to end in pCRscript (Stratagene) and inserted into the 5' and 3' ends of PHP1528 and PHP3528 to construct PHP13394 and PHP13395. PHP13396 and PHP13397 were constructed similarly using two copies of the region from -680 to -243 of *Adh1*, that does not contain MITEs.

3.2 Transformation Methods

The BMS cell line was maintained continuously from 1993 to 1998 in Dave Somers's lab (University of Minnesota, Minneapolis-St. Paul). Several sub-lines were requested from the Somers lab over the course of this study, and were designated BMS-S, BMS-S3,

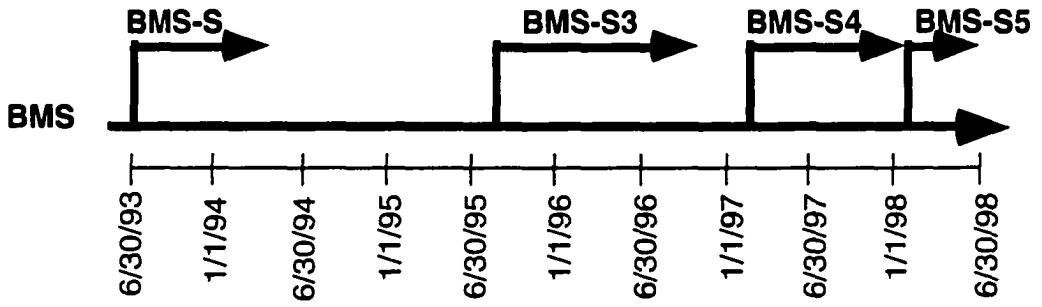


Figure 3. BMS use time-line. The original BMS line is maintained by Dave Somers lab at the University of Minnesota. Five requests for subcultures were made. Four of these subcultures were use for experiments in our lab.

BMS-S4 and BMS-S5 (Figure 3). Table 3 summarizes the use of each of these cell lines through the course of my dissertation study. The lines were subcultured twice a week using 586 media (modified MS2-D media (Bittel et al., 1996)). Prior to transformation,

Table 3. Summary of BMS subculture usage.

BMS Line	Time in Use	Number of Experiments	Number of Events Assayed
BMS-S	6/93 - 4/94	4	2298
BMS-S3	8/95 - 11/96	5	5009
BMS-S4	2/97 - 1/98	5	1326
BMS-S5	1/98 - 1/98	2	2685

cells were prepared for bombardment essentially as described in Unger et al. (1993). The procedure was optimized for the BMS cell line through cooperation with Grace St. Clair at Pioneer Hi-Bred International., Inc. BMS cell lines were subcultured two days before shooting. One day before shooting, cells were placed in osmoticum (586 medium + 3% polyethylene glycol 8000) at a density of 200 mg cells/ml. Ten nanograms of reporter plasmid and selectable marker plasmid were precipitated onto 750 μg of 1.8 μm tungsten

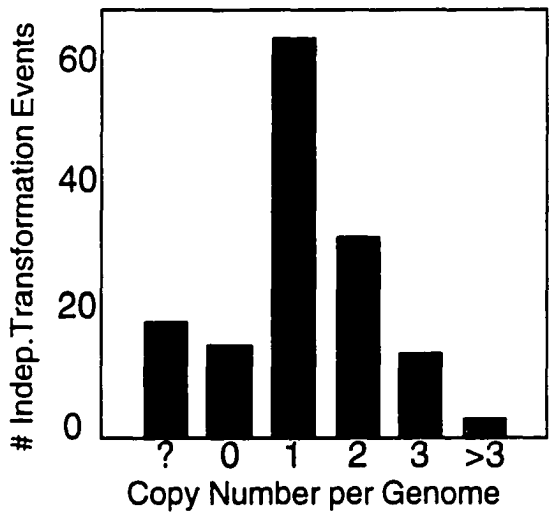


Figure 4. Copy number frequency distribution for non-selected transgenes in BMS was determined for a DNA dose of 3.33 ng DNA (equivalent amounts of non-selected reporter and selectable marker) per bombardment. The predominant copy number is one per genome. Unpublished data was provided by B. Drummond, M. Ross, D. Bond, G. St. Clair and B. Bowen (Pioneer Hi-Bred International, Inc.) ? indicates poor resolution from Southern blot analysis.

beads (General Electric), and each preparation was divided into six aliquots for bombardment. Twenty nanograms total DNA was used for all experiments, except for treatments using PHP6602, PHP7522, PHP7528, PHP8119, PHP13327, and PHP13328. For these plasmids amounts equimolar to the control plasmid PHP1528 were used. This DNA dose yields a majority of events that contain two or fewer copies of the transgene (Drummond et al., 1991 and unpublished data, see Figure 4). One half milliliter of cells were pipetted in a two centimeter circle onto sterile filter sets (consisting of a grade 391 filter (Whatman) on top, and a grade 363 filter (Whatman) beneath), premoistened with 750 µl of the same media used for osmoticum. Cells were bombarded with a PDS1000 helium gun (BioRad) using a 1100 psi rupture disk. Immediately after bombardment, cells were removed by

placing the top filter on solid 586 media containing 3% Gel-Rite without selection. Three days after bombardment, the cells were scraped off the filter, suspended in 4 ml of 586 liquid media and aliquoted (1 ml/plate) to four plates of 586 solid media containing the herbicide BASTA (AgrEvo, Wilmington, DE) at 3 mg/L. Stable transformants were recovered 4-8 weeks after bombardment and were subcultured twice to confirm BASTA resistance. Events were assayed for luciferase (or *GUS*) expression 7 days after the second subculture. Expression levels were normalized to total protein levels using the method of Bradford (1976) and reagents from Bio-Rad.

For transient gene expression assays, BMS cell lines were subcultured 24 hours before bombardment and placed in osmoticum 4 hours prior to shooting. 100 mg of cells were plated onto filters. Ten μg of the test luciferase constructs were mixed with 2 μg of PHP264 and precipitated onto 1.0 μM tungsten beads (GE or BioRad). Bombardments were done using a 600 psi rupture disc. 20 hours after bombardment, cells were harvested and assayed for luciferase and *GUS* expression. Gene expression levels were normalized by dividing luciferase expression measurements (in light units per ml) by *GUS* expression levels (in light units per μl) measured in an equivalent volume of extract.

3.3 Gene Expression Assays and Data Analysis

Luciferase assays were done essentially as described by the luminometer manufacturer (Analytical Luminescence Laboratory, 1992). 100 mg of sample callus tissue was ground in 300 μl of 0.1 M phosphate buffer, pH 7.8, and 1 mM DTT and 10% of the cleared extract was diluted with 200 μl of buffer in each assay. 100 μl of 1 mM luciferin was

added and light units were measured using a 10-sec. integration time on either a single-well (Model 2010) or a multi-well luminometer (Model 96000) from Analytical Luminescence Laboratories. The Gus Light kit from Tropix, Inc. was used to assay *GUS* expression for the transient assays. 1% of the sample extract was used, and light units were measured using a 5-sec. integration time on a single-well or multi-well luminometer, according to the manufacturer's recommended protocol. For stable transformants, light units were normalized to total soluble protein using the method of Bradford (1976) and reagents from Bio-Rad.

Two different luminometers were used in this study. Most of the reported data was obtained with the single-well luminometer (Model 2010), but some experiments were assayed on the multi-well luminometer (Model 9600) which could read microtitre plates. Although both luminometers could reliably measure differences in luciferase expression between events, measurements obtained on one instrument could not be directly compared to the other because the conversion was not linear. This is because Model 2010 uses a photon counting method and Model 9600 measures resistance across a photometer (personal communication with Analytical Luminescence Laboratories). All transgenic events from BMS-S5 were measured on the multi-well luminometer and therefore could not be directly compared with the other cell lines in Chapter IV, section 2.2. Other experiments done on the multi-well luminometer include the 1x vs. 100x DNA doses (Chapter IV, section 2.4) and the experiments comparing linearized vectors (Chapter IV, section 3.2).

3.4 Statistical Analysis

Statistical analysis of log-transformed data was done using JMP version 3.1.5 for the Macintosh, from the SAS Institute, Inc. To test for normality, I used the Shapiro-Wilk test for normality. The cumulative distribution function (cdf) was calculated with the following formula:

$$\text{CDF (point k)} = \frac{\sum_{j=1}^{k} (LU/ug)_j}{\sum_{i=1}^{n} (LU/ug)_i} \text{ where n = \# events and k=1,2,3,...,n .}$$

3.5 S-Adenosyl-L-Homocysteine Treatments

S-adenosyl-L-homocysteine (SAH) was added to media at 150 μM (De Cabo et al., 1994). Each transgenic cell-line treated with SAH was first assayed prior to treatment and then placed on plates containing SAH. Samples were taken at 48 hours and 10 days and assayed for luciferase or *GUS* expression. Trichostatin A (TSA) treatments were done similar to SAH. TSA was added to 586 solid media from a 1000x stock to a final concentration of 1.5 μM (Yoshida et al., 1995). Samples were left on treated media for 24 hours and then assayed.

3.6 *dam* Methylase Methylation Assay

Transformation of the inducible cell line 5.2 (a transgenic BMS line expressing a modified estrogen receptor (Roth et al., in prep.)) was done the same as the previous transformations with the following modifications. A single plasmid was used, containing *dam* methylase under inducible control by the estrogen response elements and 35S::MALS

which conferred resistance to chlorsulfuron (PHP7576; Figure 2O). Selection media following bombardment contained 3mg/L BASTA and 20 ppb chlorosulfuron (DuPont, Wilmington, DE).

Dam methylase expression following induction by estradiol resulted in growth arrest (data not shown). To ascertain if the *Adh1* 5' MAR was methylated by *dam in vivo*, suspension cultures of stable transformants were induced in liquid media containing 10 μM of estradiol. After 48 hours of induction, genomic DNA was prepared using the CTAB extraction method. One to two grams of homogenized tissue were added to 9 ml of CTAB buffer (0.1 M Tris, pH 7.5, 27.4 mM CTAB (hexadecyltrimethyl-ammonium bromide), 0.7 M NaCl, 0.5 M EDTA, pH 7.5) and incubated at 65° C for 1 hour. The mixture was extracted twice with chloroform and precipitated. The precipitated DNA was "hooked" with a pasture pipette and dissolved in 100 mM Tris (pH 7.5), 10 mM EDTA (pH7.5), 0.7 M NaCl by incubation at 60° C for 1 hour. The DNA was precipitated with two volumes of ethanol, rinsed in 76% ethanol and dissolved in TE (10 mM Tris (pH 7.6), 1 mM EDTA (pH 8.0)). 15 μg DNA was digested with the appropriate enzyme (*Dpn*I, *Dpn*II, or *Sau*3A (New England Biolabs, Beverly, MA) and separated on a 1% agarose gel run overnight at 1 V/cm. DNA was transferred to positive-charged nylon membrane (Boehringer Mannheim) using standard procedures (Sambrook et al., 1989). For Southern analysis, the *Adh1* 5' MAR was used as a probe. T7 and T3 PCR primers were used to amplify the *Adh1* 5' MAR from a Bluescript vector according to manufactures recommended procedure for producing digoxigenin labeled probes (Boehringer Mannheim Corporation, 1995) using a dUTP:dTTP nucleotide ratio of 1:4. Southern analysis was completed using proto-

cols outlined in the Genius System User's Guide (Boehringer Mannheim Corporation, 1995).

3.7 Nuclear Matrix Binding Assay

Nuclear matrix binding assays were done by Dr. Zoya Avramova at Purdue University essentially as described (Avramova and Bennetzen, 1993). Matrices were isolated from 0.5 A260 units of leaf nuclei. 50 ng of MAR containing plasmid, ARS1, *Adh1* 5' MAR or *Mha1* 5' MAR in pUC19, was cut with *Hin*dIII/*Eco*RI, *Hin*dIII/*Not*I, or *Hin*dIII respectively, to separate the MAR from the plasmid backbone. The DNA was random primed with ^{32}P and incubated with the isolated nuclear matrices. Varying concentrations of *E. coli* DNA (100 to 2500-fold molar excess) were used as competitor to prevent nonspecific binding (the A-T content of E. coli DNA is ~50%) and to compare the affinity of different DNA fragments for proteins in the matrix preparation. After the incubation, the 100 µl reaction was placed on a centrifuge and the nuclei were pelletted. The supernate was discarded and DNA that remained bound to the pelletted nuclear matrices was run on a gel, transferred to a nylon membrane and exposed to film.

CHAPTER IV. RESULTS AND DISCUSSION

4.1 *In Vitro* Properties of Matrix Attachment Regions Do Not Correlate With Their *In Vivo* Effects

4.1.1 Affinity of MARs for maize nuclear matrix *in vitro*

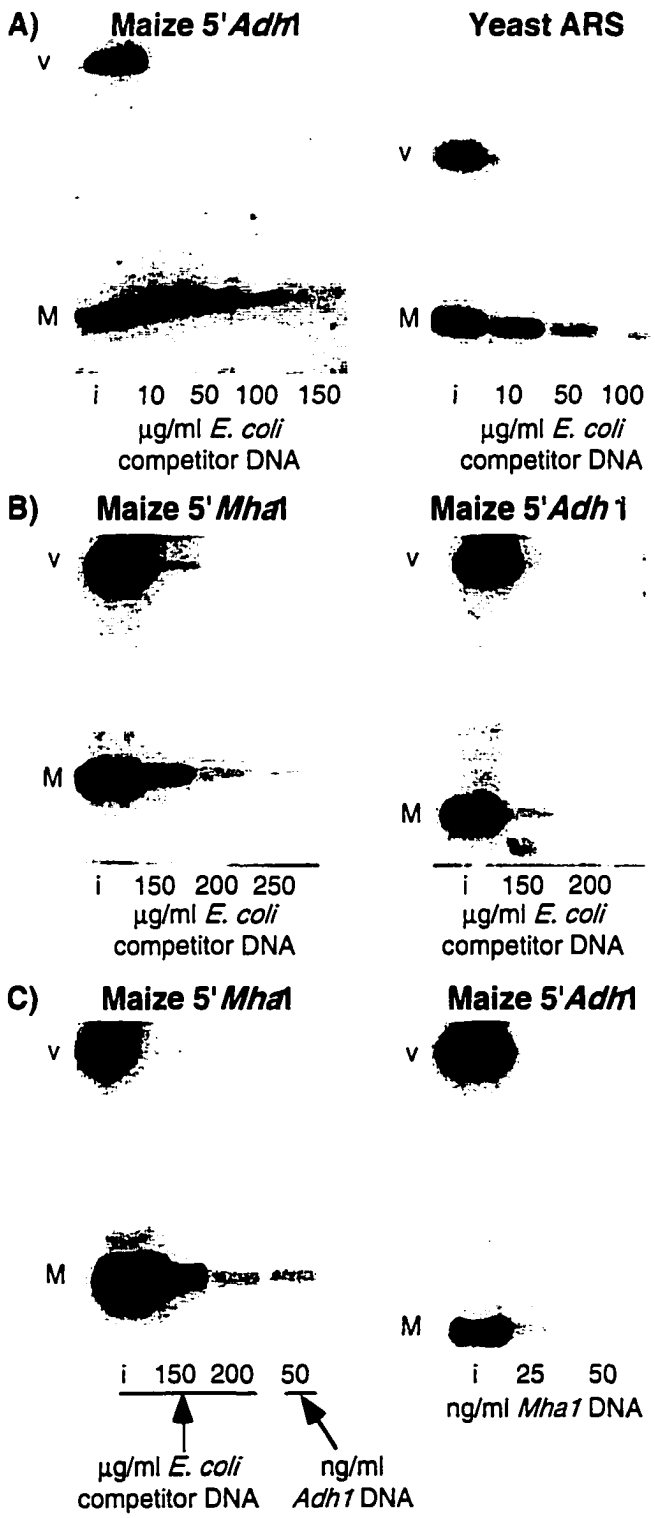
In this study, we used three MAR elements: yeast ARS1(Amati and Gasser, 1988), and two MARs from the 5' regions of maize *Adh1* and *Mha1*, respectively (Avramova and Bennetzen, 1993; Jin and Bennetzen, 1994). Figure 5 shows the results of binding assays with maize nuclear matrix preparations, performed as described in Section 3.7. For each MAR (M), the binding to the maize nuclear matrix was more specific than the binding of the vector (V) sequences included in each assay. The *Mha1* 5' MAR displayed the highest affinity for binding to the nuclear matrix, and the yeast ARS1 sequence bound less efficiently than either of the maize MARs (Figure 5A and B). In the presence of a large excess of *E. coli* competitor DNA (≥150 μg/ml), approximately 10 times more *Mha1* 5' MAR remained bound compared to *Adh1* 5' MAR. Inhibition of *Adh1* 5' MAR binding by the addition of cold *Mha1* 5' MAR and *vice-versa* (Figure 5C) confirmed the relative strength of binding by each MAR: 50 ng/ml of cold *Mha1* 5' MAR completely abolished *Adh1* 5' MAR binding, whereas 50 ng/ml of cold *Adh1* 5' MAR left 9% of labeled *Mha1* 5' MAR bound to the matrices (Figure 5C). Because *Adh1* and *Mha1* 5' MARs can compete with one another in binding maize nuclear matrices, some of the components required for matrix binding of *Adh1* 5' MAR must also be involved in binding the *Mha1* 5' MAR.

Figure 5. MAR Binding Assays. In all panels, vector and MAR fragments are labeled V and M, respectively. Labeled DNA, without binding matrices, is shown in lane i.

A) Yeast ARS1 sequences bind maize matrices with a lower affinity than the maize *Adh1* 5' MAR. In the presence of a 1000-fold molar excess of competitor DNA (100 μg/ml), 3% of yeast ARS1 and 10% of the *Adh1* 5' MAR remained matrix-bound.

B) The *Mha1* 5' MAR displayed the highest affinity for binding to the matrix. In the presence of a 1500-fold molar excess of competitor (150 μg/ml), 30% of the *Mha1* 5'MAR remained bound, while under the same conditions, only 3.5% of *Adh1* 5' MAR remained bound.

C) Binding of *Mha1* 5'MAR in the presence of different concentrations of *E. coli* DNA or 50 ng/ml of unlabeled *Adh1* 5' MAR (left panel). 9% of *Mha1* 5'MAR is still bound to the matrix in the presence of 50 ng/ml of unlabeled *Adh1* 5' MAR. When the binding of labeled *Adh1* 5' MAR was challenged with different concentrations of unlabeled *Mha1* 5'MAR (right panel), 25 ng/ml of the specific competitor left only 3% of the *Adh1* 5' MAR bound to the matrix and 50 ng/ml of unlabeled *Mha1* 5'MAR completely abolished the matrix-binding of the *Adh1* 5' MAR.


A)
Maize 5'Adh1
Yeast ARS
v
M
i 10 50 100 150
µg/ml E. coli
competitor DNA
i 10 50 100
µg/ml E. coli
competitor DNA
B)
Maize 5'Mha1
Maize 5'Adh1
i 150 200 250
µg/ml E. coli
competitor DNA
i 150 200
µg/ml E. coli
competitor DNA
C)
Maize 5'Mha1
Maize 5'Adh1
i 150 200 50
µg/ml E. coli
competitor DNA
ng/ml
Adh1 DNA
i 25 50
ng/ml Mha1 DNA

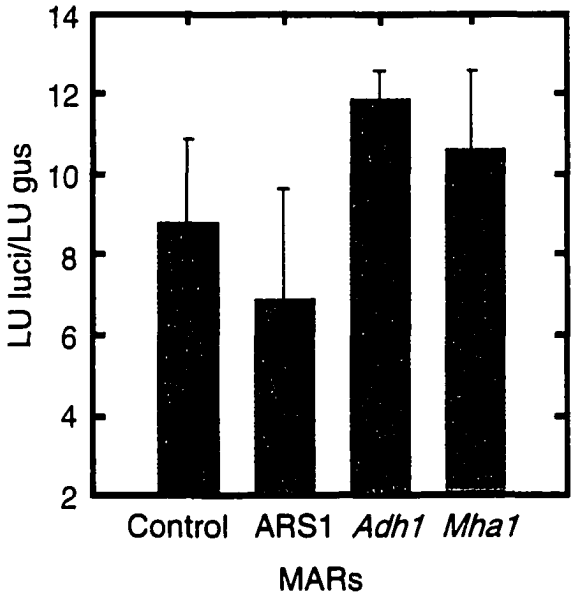


Figure 6. Effects of MARs on transient expression of 35S::luciferase. Five reps for each treatment were assayed for luciferase and *GUS* expression. 35S::luciferase vectors PHP1528, PHP5483 (ARS1), PHP6248 (*Adh1*), and PHP6486 (*Mha1*) were mixed 5:1 with 35S::*GUS* vector PHP264. Relative levels of gene expression were calculated by normalizing luciferase to the level of *GUS* expression. F-test indicated no significant difference between means (P=0.21).

4.1.2 MAR elements do not affect expression prior to integration in maize BMS cultures

Transient assays of reporter gene expression were used to test if each MAR has enhancer activity in BMS cells (Allen et al., 1993; Mlynarova et al., 1994; Poljak et al., 1994; Allen et al., 1996). Since stably integrated transgenes flanked with MARs have often been shown to have higher levels of expression than unflanked transgenes, it is important to determine by transient assays how much of this increase in gene expression conferred by MARs is due to an enhancer effect that would be observed prior to integration. Figure 6 compares the expression level of constructs in which 35S::luciferase was flanked by each of the three MARs studied with the control vector lacking MARs (PHP1528). The

luciferase constructs being tested were introduced into BMS-S calli in combination with a fixed level of a 35S::*GUS* plasmid (PHP264), and luciferase expression was normalized to *GUS* expression. None of the three MAR elements we tested increased or decreased the expression level to a large extent compared to the control vector without MARs. Thus, none of the MARs appears to enhance the expression of unintegrated genes *in vivo*.

4.1.3 Transgene expression levels in stable BMS transformants are bimodally distributed

To facilitate the study of a large number of BMS transformants, we chose the *BAR* gene as a selectable marker and firefly luciferase (Luci) as a reporter. Luciferase is simple to assay and is measurable over a range of 5 orders of magnitude. 35S::*BAR* and 35S::luciferase genes were introduced in *trans*- on separate plasmids at a dosage (3.3 ng/ bombardment) that had been shown previously to produce an average copy number of 1-3 transgenes per transformant (Drummond et al., 1991) B. Drummond, M. Ross, D. Bond, G. St. Clair and B. Bowen, unpublished data; Figure 4). In one treatment, neither the reporter nor selectable marker contained MARs, whereas, in the other treatment, both the reporter and selectable marker were flanked with one of the three MAR elements.

Because of the enormous variation typically seen in plant transgene expression levels, it is common to do a log transformation of the data prior to analysis (Nap et al., 1993). Some transformation studies have reported expression level data that are normally distributed after log transformation (Mlynarova et al., 1994; Allen et al., 1996), whereas others have reported a non-log-normal distribution (Hobbs et al., 1990; Breyne et al., 1992). Analysis of variance and other statistical tests can be applied if the data are normal (or log-

normal), but nonparametric statistical analyses must be applied, if the data cannot be normalized by transformation.

Figure 7 shows log-transformed luciferase expression level data among BMS calli transformed with each of the four vector combinations tested. When more than 50 events per treatment were analyzed, it became clear that transgene expression levels were bimodally distributed; with lower sample sizes, the distribution was not significantly different from log-normal. This was true for all treatments including the controls lacking MAR elements. Because the data are not normally distributed, I could not use analysis of variance to compare treatments.

4.1.4 MAR effects on transgene expression *in vivo* do not correlate with *in vitro* binding strength

The ARS1 MAR (Figure 7C) and the *Adh1* 5' MAR (Figure 7D) each increased the average luciferase expression level by 5.8- and 26.8-fold, respectively, compared to the control (Figure 7A). The increase in the level of average expression was associated with a shift from the first peak of the bimodal graph to the second peak (compare Figure 7C and D with A). This shift from a majority of low expressing events to high expressing events was largely responsible for the increase in average expression because the overall range of expression levels was similar in all treatments. The *Mha1* 5' MAR data (Figure 7C) did not show this shift and increased average luciferase expression by only 1.5-fold compared to the control. Previously, Allen et al. (1996) showed that a MAR with greater affinity for matrix preparations was able to increase expression of transgenes to a greater extent than a more weakly binding MAR. However, my results show that although the *Mha1* 5' MAR

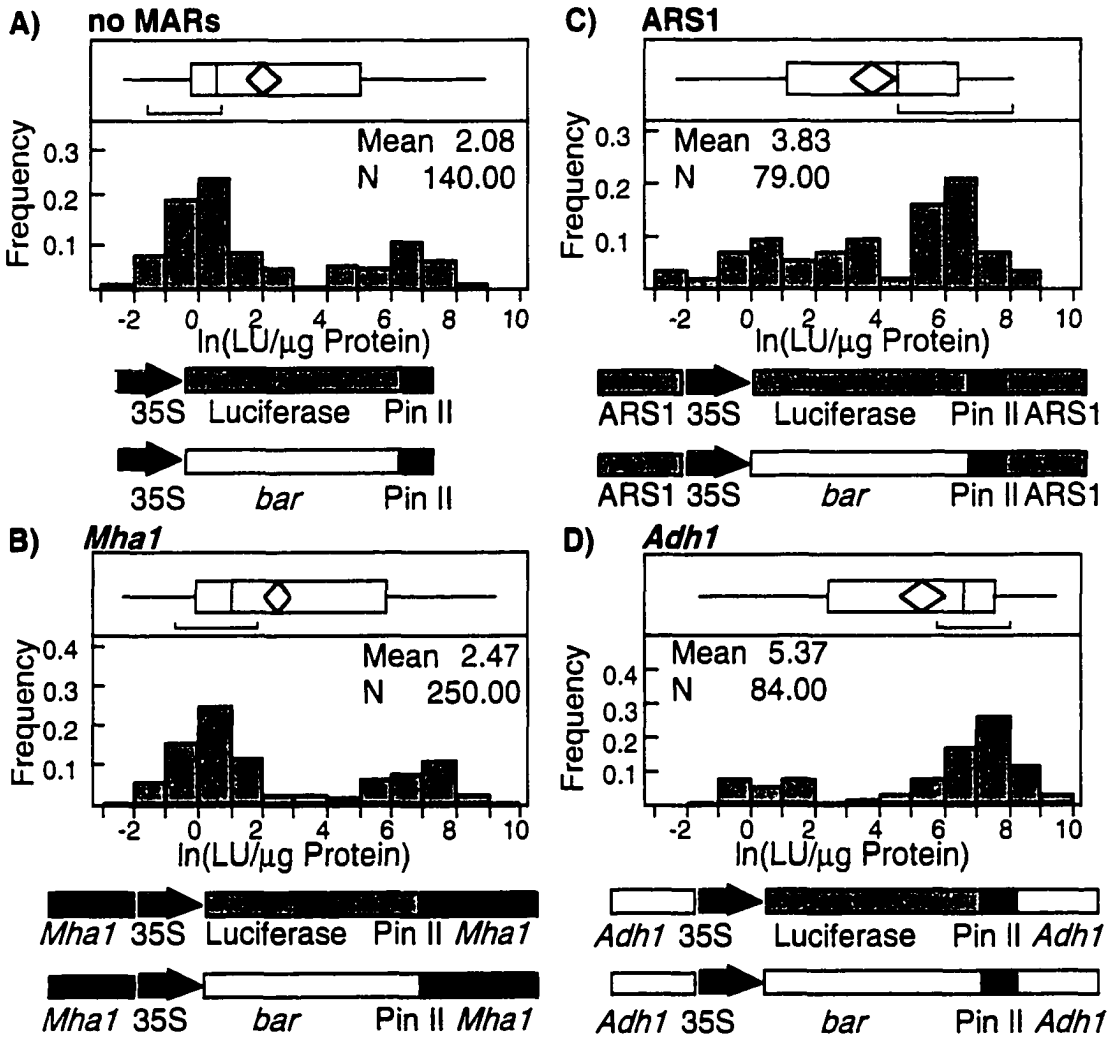


Figure 7. The plasmids shown were shot *in trans*, one with luciferase and the other with *BAR*. Transgene expression level distributions are shown in the histograms. The box above the histograms is an outlier box plot. The box represents the interquartile range, or the difference between the 25th and 75th quantiles. The "whiskers" represent the range (computed as 150% of the interquartile range). Points outside the whiskers are possible outliers. The vertical line inside the box represents the median, and the diamond represents the mean. The bracket underneath the box identifies the most dense 50% of all the observations. All treatments were bimodal and the shape of the distributions are similar for no-MARs and *Mha1*, indicating *Mha1* had no effect on expression. ARS1 and *Adh1* both increased the expression level and shifted the majority of expressing events to the higher-expressing peak of the bimodal distribution.

has a greater affinity for maize matrices than the *Adh1* 5' MAR, the *Adh1* 5' MAR significantly increased transgene expression, whereas the *Mha1* 5' MAR had almost no effect. ARS1, the weakest binding MAR of the three studied, was still able to increase average luciferase expression levels 5.8 fold. This was a significant increase, although not as great as the 24-fold increase that Allen et al. (1993) found for ARS1 in tobacco.

4.1.5 *Adh1* 5' MAR and ARS1 prevent transgene silencing in BMS

Because the histograms in Figure 7 only include data from expressing events, results were also displayed using the cumulative distribution function (cdf). This graphical method effectively summarizes all the data from both expressing and non-expressing events. The cdf graphs, shown in Figure 8, reveal that flanking luciferase with either the *Adh1* 5' MAR or ARS1 increased the proportion of events that express the luciferase transgene, whereas flanking luciferase with the *Mha1* 5' MAR had no effect on the transgene expression level distribution.

Figure 9 displays the differences seen among the proportions of non-, low-, and high-expressing events when the MAR-containing events were compared to the control. We divided events into low and high expressors by arbitrarily selecting a threshold between the two peaks of each bimodal distribution (Figure 7). The graph shows that ARS1 and the *Adh1* 5' MAR more than doubled the number of high expressing events. ARS1 increased the number of high expressing events primarily at the expense of the non-expressing events, whereas the *Adh1* 5' MAR nearly tripled the number of high expressing events at the expense of both non- and low-expressing events. In contrast, the *Mha1* 5' MAR had

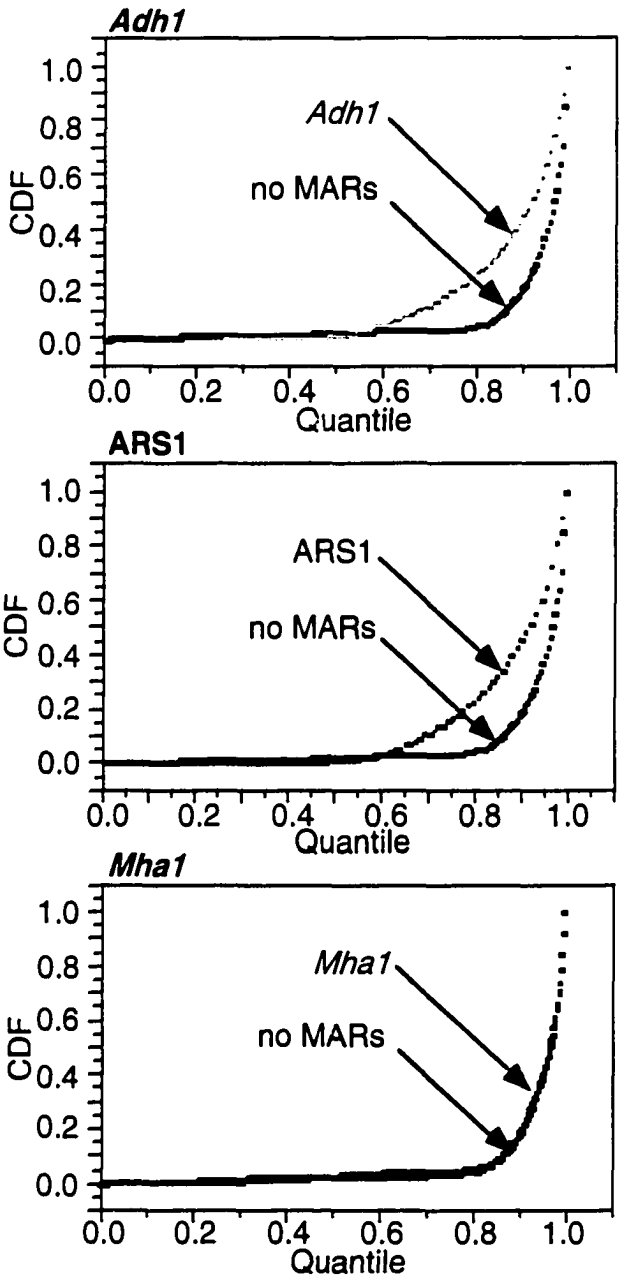


Figure 8. Cumulative distribution function graphs. Flanking 35S::luciferase with either the *Adh1* 5' MAR or ARS1 resulted in a significantly different expression distribution, whereas flanking 35S::luciferase with the *Mha1* 5' MAR had no effect.

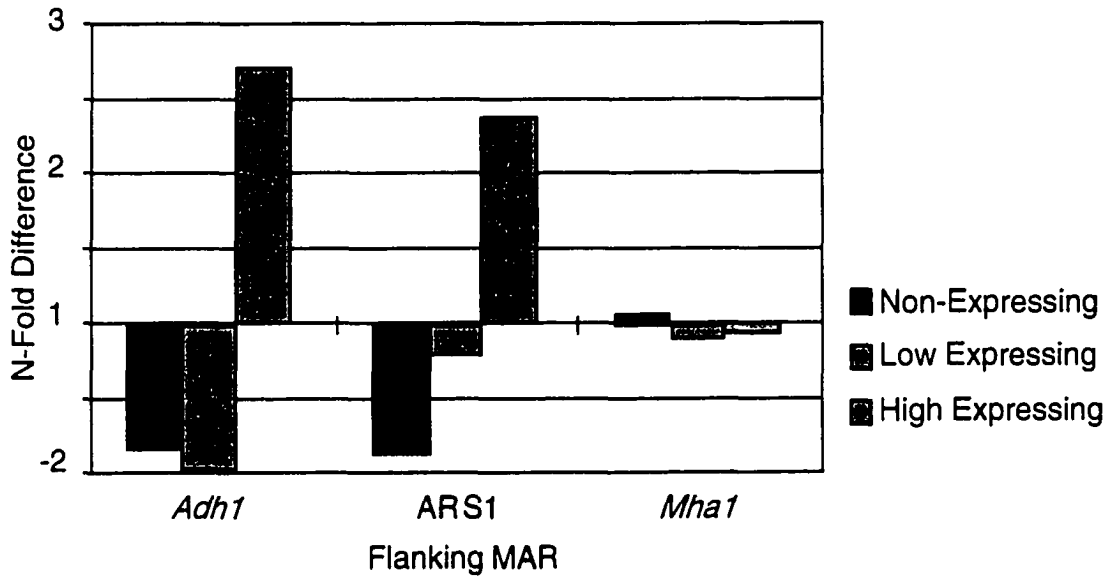


Figure 9. N-fold difference graph. Values indicate the n-fold difference from the control without MARs. Main effect of ARS1 and *Adh1* MARs is to increase the number of high expressing events. *Mha1* had no effect on expression levels.

very little effect on the proportions of non-, low-, or high expressors compared to the control. Both ARS1 and *Adh1* 5' MAR decreased the number of non-expressors approximately 2-fold, indicating that both of these MARs increase average expression by preventing transgene silencing.

4.1.6 Discussion

The results of this section are summarized in Table 4. The three MAR elements tested each had different binding affinities for maize nuclear proteins, representing an approximately 30-fold difference in binding strength. Allen et al. (1996) suggested that there is a correlation between MAR binding strength and the effects of MARs on transgene expression. They found that the tobacco *Rb7* MAR, which has a greater affinity for tobacco

Table 4. Summary of Results.

MAR	Relative Binding Strength	Mean Expression Level[a]	N-Fold Increase Over Control	N-Fold Increase in High Expressors	Range[b]
no MAR	N/A	8.0	1.0X	N/A	11.24
ARS1	1	20.7	5.8X	2.4X	10.44
Adh1 5'	3	214.9	26.8X	2.7X	11.17
Mha1 5'	30	11.8	1.5X	1.0X	11.52

a. LU/μg Protein

b. 1.5 * interquartile range (same as whiskers in Figure 7) expressed as ln(max.)-ln(min.) in ln(LU/μg Protein)

matrixes than the yeast ARS1 MAR, increased reporter gene expression 140-fold compared to a 24-fold increase with ARS1. In this study, I found that the stronger binding MAR (*Mha1* 5' MAR) had no effect on reporter gene expression, but a weaker binding MAR (*Adh1* 5' MAR) increased the average transgene expression level by 26.8-fold and the likelihood of obtaining a high expressing event by 3-fold. In contrast to the suggestion made by Allen et al. (1996), I found no correlation between MAR binding strength and MAR effects on transgene expression *in vivo*.

Both ARS1 and the *Adh1* 5' MAR had similar effects on stable transgene expression distributions, but the *Adh1* 5' MAR had a greater effect on average transgene expression levels. Both MARs increased mean expression level in stable transformants but not in transient assays. Neither MAR appeared to reduce the range of expression (Table 4), indicating that position effects on transgene expression level variation were not affected. Both MARs increased the proportion of expressors at the expense of non-expressors, however, indicating that the main effect of each MAR was to decrease the likelihood of transgene silencing. One practical benefit suggested by our data, is that two to three times fewer

transformants might need to be generated and/or screened when a transgene is flanked by either ARS1 or the *Adh1* 5' MAR. This conclusion was based on data obtained with a single subculture of BMS (BMS-S) over a period of 12 months. Subsequent study of MAR effects with different transgenes and in different BMS subcultures made me modify these conclusions, as described more fully in the next section.

Recent studies of the 5' and 3' MARs from the tomato *Heat Shock Cognate 80* (*HSC80*) gene suggest a possible reason for the differences observed for the *Adh1* and *Mha1* 5' MARs in our work (Chinn and Comai, 1996; Chinn et al., 1996). These studies indicated that the 5' and 3' MAR elements were essential for regulated expression, but only when combined with transgenes that harbored introns of *HSC80*. Absence of either MAR or any of the introns reduced or eliminated expression. All of my constructs used in this study contained the first intron of maize *Adh1*, because this element significantly enhances maize transgene expression (Callis et al., 1987). Thus, cooperation between factors bound by the *Adh1* 5' MAR and the *Adh1* first intron could be important for the *Adh1* 5' MAR effect *in vivo*. In contrast, factors bound by the *Mha1* 5' MAR may not be compatible with factors bound by the *Adh1* intron. To explore this hypothesis further, it would be interesting to test the *Mha1* 5' MAR with other *Mha1*-derived sequences (e.g. introns) and the *Adh1* 5' MAR without the *Adh1* first intron.

All three MARs bound maize nuclear matrix proteins *in vitro*, and some of the factors that bound the *Adh1* 5' MAR also bind the *Mha1* 5' MAR (Figure 5). However factors which bind both *Adh1* and *Mha1* 5' MARs *in vitro* are clearly not sufficient for the *Adh1* 5' MAR effects *in vivo*.

Yeast ARS1 can bind to tobacco matrices *in vitro* and when used to flank transgenes in tobacco callus, raised transgene expression levels 24-fold (Allen et al., 1993). In collaboration with Zoya Avramova, I have demonstrated that ARS1 also binds maize matrices *in vitro*. In maize, however, ARS1 had less of an effect on transgene expression levels than in tobacco. Yeast ARS1 also had less of an effect on expression levels than an endogeous MAR which is consistent with the findings of Allen et al. (1996) in tobacco. If MAR elements participate in a regulatory role through the binding and action of various proteins, it might be expected that an endogenous MAR would bind these proteins more readily and thereby have a greater effect on the level of transgene activity.

Do the maize *Adh1* 5' MAR and yeast ARS1 prevent transgene silencing by acting as boundary elements? Boundary elements block neighboring control elements, such as enhancers or silencers, and prevent the spread of heterochromatin (Geyer, 1997). This should lead to copy number dependent and position independent expression. Several studies in plants have reported decreases in variation when flanking a reporter gene with MAR elements, suggesting limited boundary element function (Breyne et al., 1992; Mlynarova et al., 1994; van der Geest et al., 1994; Mlynarova et al., 1996), and in one case, partial copy number dependent expression (Schöffl et al., 1993). However, none of the MAR elements in these studies were completely able to overcome position effects. In my study, the maize *Adh1* 5' MAR and yeast ARS1 had no effect at all on reducing position effects (Table 4). Although these MARs may prevent transgene silencing by impairing the establishment of heterochromatin or a repressed transcription state, they appear to have no abil-

ity to block neighboring control elements such as silencers or enhancers. Thus, neither MAR has the properties of an insulating boundary element as defined by Geyer (1997).

What could be responsible for the MAR effect *in vivo*? One possibility is that MARs may create chromatin loops that are accessible to factors required for initiation of transcription, but that the level of steady-state transcription is determined by nearby regulatory elements. Conversely, MARs may function as regulatory elements responsible for the transition from silent to active chromatin either through their inherent unwinding capability or by binding regulatory proteins that affect the likelihood that an active transcription state is established. This second possibility is consistent with the bimodal distribution of gene expression levels and the effect of MARs in shifting the expression level distribution in favor of high expressors.

What are possible reasons for the bimodal distributions of the expression level data? Hobbs et al. (1990) reported bimodal expression in tobacco transformed with *GUS*. They found that when transformed tobacco plants contained multiple copies of the transgene, the expression was much lower than in tobacco plants containing a single insert. Allen et al. (1996) reported that transgenic events containing greater than 10 copies had a greatly reduced expression level than events with less than 10 copies. The fact that multiple copies of a sequence in plants can trigger silencing has been well documented (Matzke and Matzke, 1995; Jorgensen et al., 1996; Wolffe, 1997).

In my study, I was unable to complete Southern blot analysis of a large number of transformants to see if copy number differences could be responsible for the bimodal distribution. However, previous data indicates that more or less all BMS transformants

obtained with the dose of DNA used here were low copy (Figure 4). Another possible explanation could be differences in methylation. Many studies have found that silenced or low-expressing transgenes were methylated and high-expressing transgenes were unmethylated (Hobbs et al., 1990; Kilby et al., 1992; Tsanev et al., 1993). Differences in the level of transgene expression and/or methylation could reflect the chromosomal position of transgene integration. For example, transgenes integrated near telomeres, centromeres, or other hetrochromatic regions may be localized in transcriptionally inactive domains of the nucleus, close to the nuclear periphery, whereas transgenes integrated at other chromosomal positions may localize in transcriptionally active domains within the interior of the nucleus. Finally, it is also possible that there are differences intrinsic to cells when the transgene transcription state is established (Pillus and Rine, 1989; Loo and Rine, 1995). For example, the probability of transgene silencing may vary in different stages of the cell cycle (Loo and Rine, 1995). Some of these possibilities are addressed in the next section.

4.2 Effects of MARs on Establishment and Heritability of Stable Transcription States Require Silencing Factors that are Lost in Aging Maize Cells

4.2.1 Non-autonomous and synergistic effects of *Adh1* 5' MAR on reduction in transgene silencing

In section 4.1, I showed that the *Adh1* 5' MAR had the greatest effect on transgene expression, so I decided to study the effects of this MAR in greater detail. Other studies of MAR elements in plants have flanked the reporter transgene, but not the selectable marker (Breyne et al., 1992; Allen et al., 1993; Allen et al., 1996). To compare my results with these studies, I tested combinations where one or the other or both transgenes were flanked

with the *Adh1* 5' MAR. As shown in the previous section, flanking both luciferase and *BAR* with the *Adh1* 5' MAR increased the average expression level significantly over that of the unflanked controls (Figure 10). However, flanking either luciferase or *BAR* alone also increased average luciferase expression levels above that of the control. These results indicate that the *Adh1* 5' MAR can increase the average expression level of 35S::luciferase non-autonomously, i.e. even when it is present on a separate vector in *trans*-. Additional copies of the *Adh1* 5' MAR flanking the *BAR* gene can apparently interact with MARs flanking luciferase and have a synergistic effect on luciferase expression.

Since transgenes introduced on separate vectors typically integrate into a site together (Czernilofsky et al., 1986; Huang and Dennis, 1989), it is possible that an unflanked transgene could become MAR-flanked by integrating between two MAR-flanked transgenes. While this could account for the effects seen when one transgene is flanked but not the other, it cannot easily account for the synergistic effect seen when both transgenes areflanked by MARs (Figure 10 and 11). Another possible explanation is that the *Adh1* 5' MAR can act as an enhancer of transcription. Thus, the MAR flanking one transgene in treatments 2 and 3 could enhance expression of both the flanked and unflanked genes. When both transgenes are flanked, the enhancer may act synergistically if cooperative binding of *trans*-acting factors is affected by extra copies of the MAR. This explanation would be appealing, except for the fact that the *Adh1* 5' MAR had no enhancer activity in the transient assays reported in section 4.1.

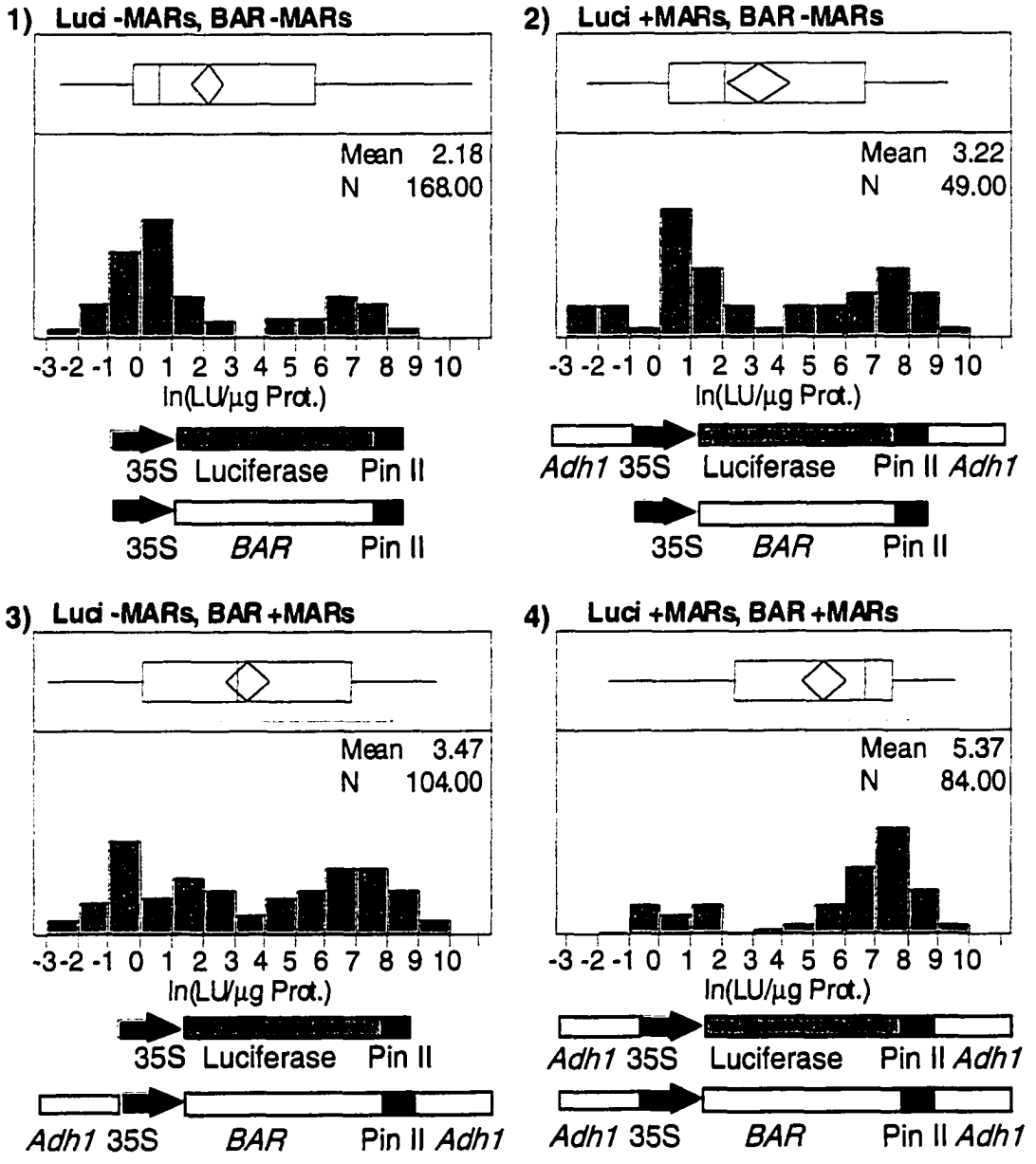


Figure 10. Histograms of *Adh1* 5' MAR in BMS-S. Treatments 1 and 4 were shown earlier comparing the effects of different MARs. In treatments 2 and 3, the effect of flanking one transgene with the MAR is midway between no flanking MARs and flanking both transgenes.

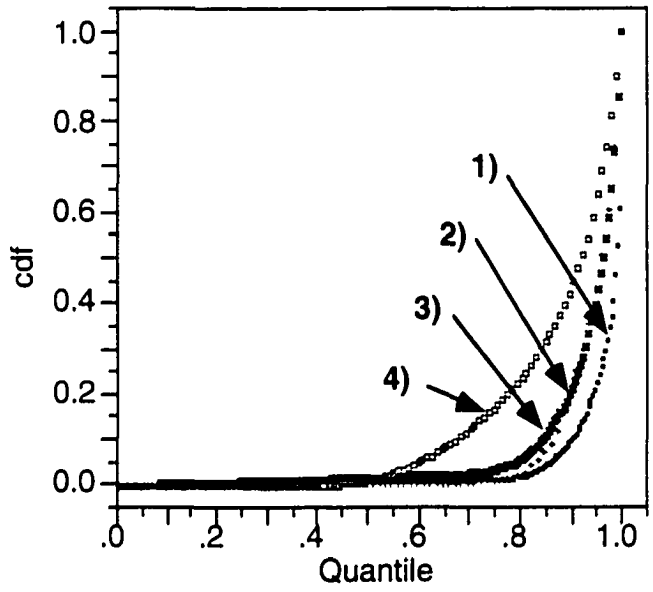


Figure 11. Cumulative distribution function graph of the data in Figure 10. *Adh1* 5' MAR flanking one transgene or the other results in distributions midway between those shown previously where both of the transgenes or neither of the transgenes were flanked by the *Adh1* 5' MAR. Numbers represent treatments shown in Figure 10.

4.2.2 *Adh1* 5' MAR prevents transgene silencing by interacting with *trans*-acting silencing factors that disappear the longer the BMS cells have been maintained

To determine if the effects of the *Adh1* 5' MAR on transgene expression levels are an intrinsic property of the sequence and stable in different cell types, I compared the behavior of 35S::luciferase flanked by this sequence in many replicated experiments using four different subcultures of BMS (BMS-S, BMS-S3, BMS-S4, and BMS-S5) that were separated from the parent cell line at different intervals over a period of five years (Figure 3). Results are summarized in Figure 12.

As described in section 4.1, the average expression level of luciferase was 26.8 fold higher for *Adh1* 5' MAR flanked events than the unflanked events in BMS-S. In BMS-S3

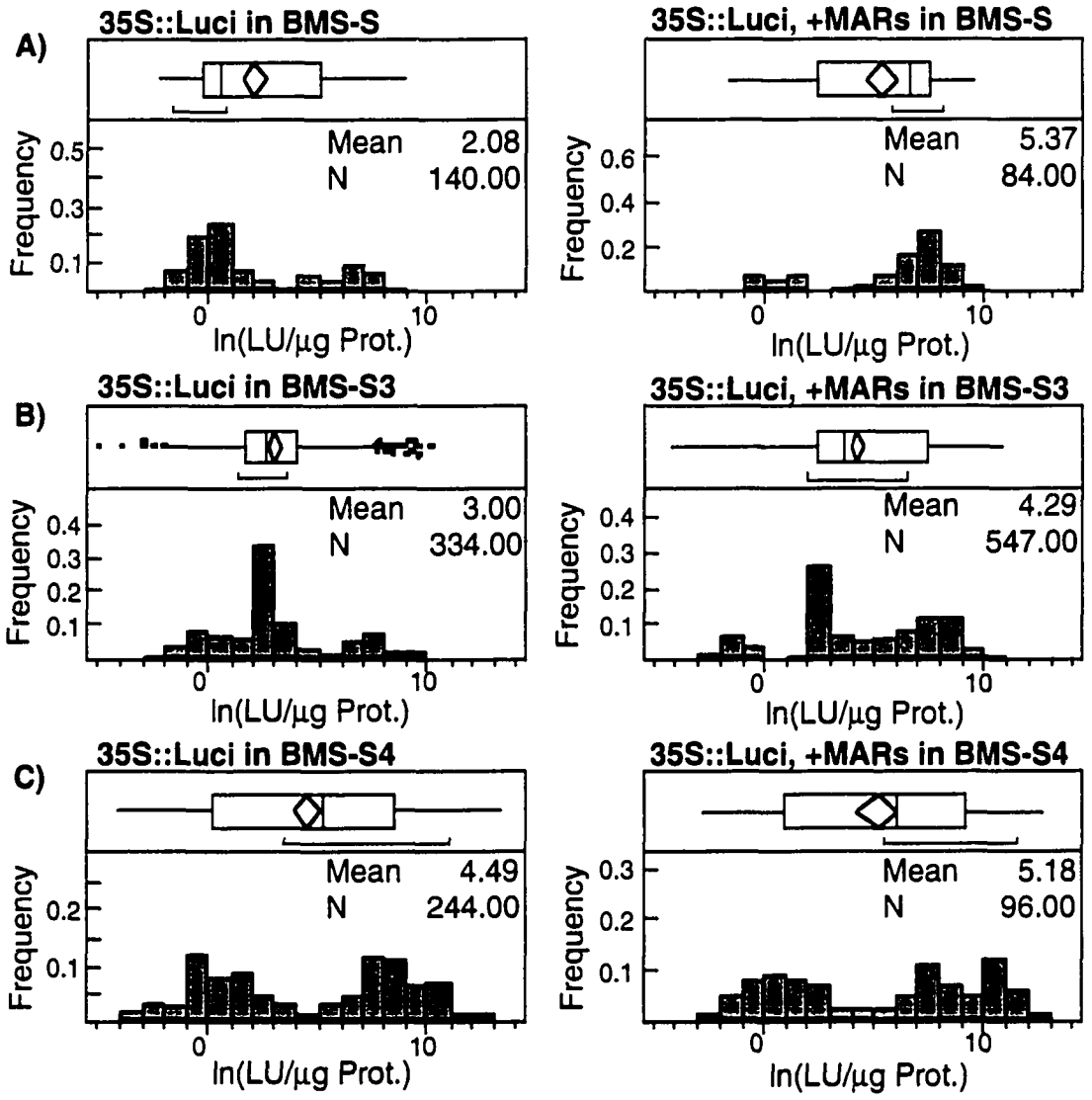


Figure 12. Histograms comparing BMS lines for *Adh1* 5' MAR-flanked and non-MAR events. A non-MAR flanked plasmid (PHP1528) or a MAR-flanked plasmid (PHP6248) was transformed into each of the BMS lines. See Figure 7 for a description of the parts of the graph. A) In BMS-S there is a distinct bimodality to the distribution of expression with the majority of expressing events in the lower peak for non-MAR events and the upper peak for MAR events. B) In BMS-S3 the distribution is similar to BMS-S except for a large peak. C) In BMS-S4 the distribution has changed so that both non-MAR and MAR events have a similar distribution. However, in all BMS cell lines the MAR increases the average expression level (26.8-fold for BMS-S, 3.6-fold for BMS-S3 and 2-fold for BMS-S4).

the *Adh1* 5' MAR increased average luciferase expression 3.6-fold (Figure 12B), and the distinction between the expression level distributions for MAR-flanked and control 35S::luciferase was not as great as had been seen in BMS-S (Figure 12A). In BMS-S4, the *Adh1* 5' MAR conferred only a 2-fold increase in the average expression level and the expression level distributions were almost identical (Figure 12C).

Figure 13 summarizes how the differences between non-MAR and MAR flanked events changed as BMS got older. Figure 13A shows how the MAR affected the proportions of non-, low-, and high-expressors in each cell line, whereas Figure 13B contrasts the varying effects of the MAR on each expression class as a function of the cell-line subculture (see Figure 3). Clearly, the effect of the *Adh1* 5' MAR on reducing transgene silencing decreased progressively over time between BMS-S, BMS-S3 and BMS-S4 (Figure 13A). Furthermore, the diminishing increase in high expressors over time was closely mirrored by smaller decreases in non-expressors and low expressors over time (Figure 13B).

Figure 14 helps clarify why the initial effect of MARs on increasing high-expressors disappeared over time. The proportion of high expressors in events transformed with the control 35S::luciferase vector lacking MARs (PHP1528) increased in each cell line (BMS-S to BMS-S3 to BMS-S4), indicating that the level of silencing in each new cell line was less than in the previous one. At the same time the proportion of high-expressing events obtained with the MAR-flanked 35S::luciferase vector (PHP6248) decreased. Hence, in BMS-S4, the proportions of non-, low- and high-expressors was essentially the same for events with or without flanking MARs. Figure 15 illustrates this in cdf format.

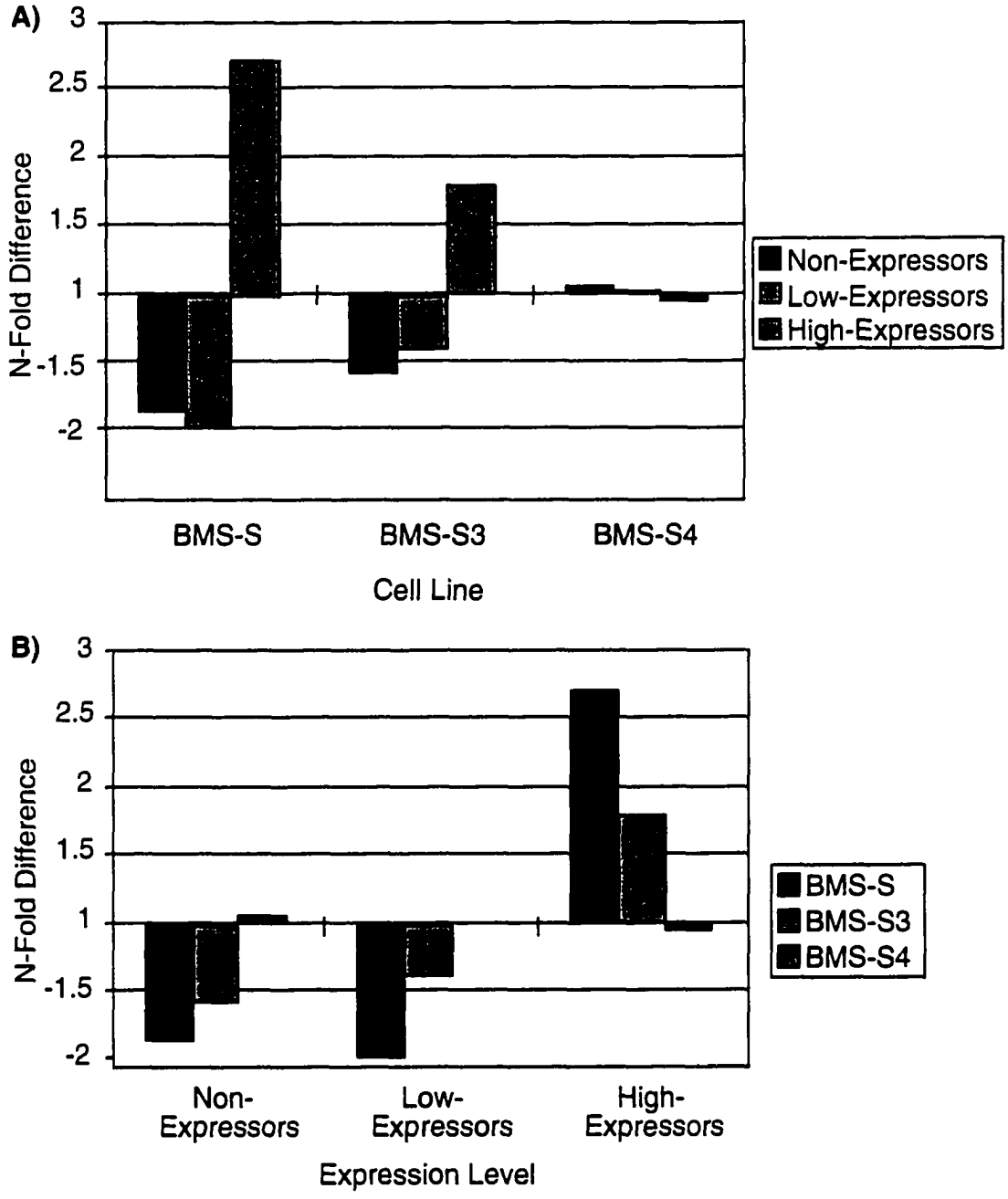


Figure 13. N-fold difference graphs of the differences in expression levels for three cell lines. A is grouped by cell line and B is grouped by expression level. Classification of non-, low- and high-expressing events was made in the same way as described in section 4.1.5.

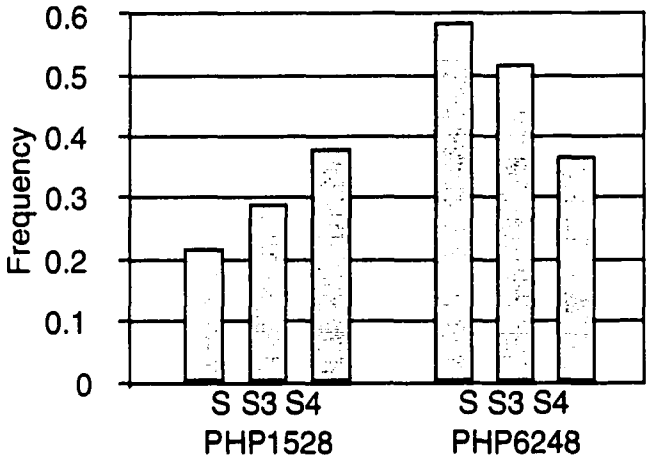


Figure 14. Graph of the frequency of high-expressors for 35S::luciferase (PHP1528) and 35S::luciferase flanked with *Adh1* 5' MAR (PHP6248) in three BMS subcultures: BMS-S, BMS-S3, and BMS-S4. The high expressors were divided as described in Section 4.1.5. In each cell line the frequency of high expressing events increases for the non-flanked control, and decreases for the MAR-flanked luciferase events.

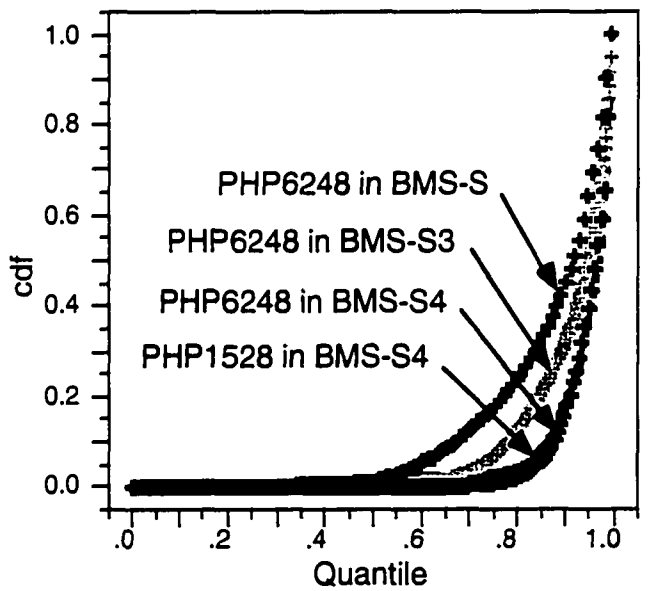


Figure 15. Cumulative distribution function graph comparing results for MAR-flanked and non-MAR events using luciferase as a reporter. For non-MAR events only those for BMS-S4 are displayed. All other non-MAR events overlap the distribution of non-MAR events in BMS-S4. For MAR-flanked events the distributions became more similar to that of the non-MAR events with each new cell line.

The cdf graphs show how the expression distribution of the MAR-flanked events changed in each cell line. The distribution for the MAR-flanked events in BMS-S shows the great est difference from the non-flanked events in BMS-S4. This difference decreases in BMS-S3, and disappears for MAR-flanked events in BMS-S4.

From these data, I conclude that the *Adh1* 5' MAR effect seen in BMS-S was not an intrinsic effect, i. e. the MAR effect must be the result of an interaction between the MAR sequence and *trans*-acting factors in a given cell type. Because BMS-S, S3 and S4 are, in theory, genetically identical, the influence of the *Adh1* 5' MAR on transgene expression must be viewed as an epigenetic effect. Furthermore, the phenotypic outcome of flanking transgenes with this MAR is unstable over time because of a MAR by cell-type interaction, and the effects of the *Adh1* 5' MAR would be predictable only when *trans*-acting variables in these sub-lines have been characterized in greater detail. Additional inferences that can be made are discussed further in Section 4.2.8.

4.2.3 *Adh1* 5' MAR prevents silencing of luciferase, but not *GUS*

To see if the *Adh1* 5' MAR effects on luciferase expression were generalizable, I decided to test whether another transgene was similarly affected. Recent reports have indicated that β-glucuronidase (*GUS)* is susceptible to epigenetic silencing inside plant cells (Baulcombe and English, 1996; English et al., 1996), which encouraged me to investigate the effects of flanking *35S::GUS* with the *Adh1* 5' MAR. These data are summarized in Figure 16.

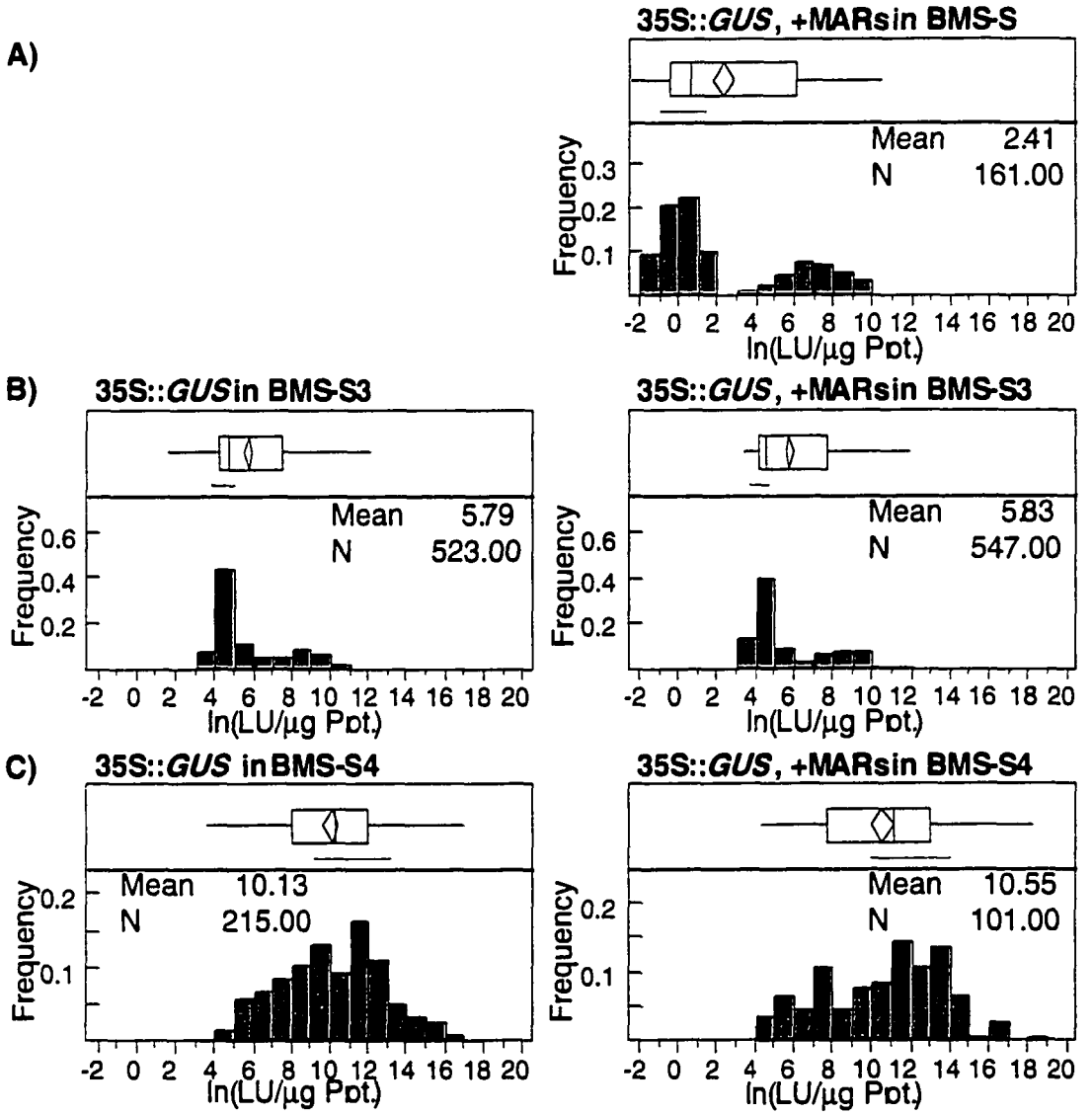


Figure 16. Histograms comparing BMS lines for MAR-flanked and non-MAR events. A non-MAR flanked plasmid (PHP264) or a MAR-flanked plasmid (PHP7974) was transformed into each of the BMS lines. See figure 3 for a description of the parts of the graph. The shape of the distributions is approximately the same for MAR-flanked events in BMS-S (A) and BMS-S3 (B) except that the mean is higher. The distribution and the means are basically the same between the non-MAR and the MAR-flanked events in BMS-S3 (B). For BMS-S4 (C) the distributions and means are again almost the same, except now the shape of the distribution is less bimodal.

35S::*GUS* vectors +/- MARs were introduced into BMS-S, but because of technical difficulties, I was only able to obtain expression level data from the MAR flanked events (Figure 16A). The expression levels were bimodally distributed as seen with luciferase. In contrast to the distribution seen for 35S::luciferase flanked by MARs (Figure 12), however, there was a significant shift in expression towards the lower peak. This suggested that 35S::*GUS* might be more susceptible to silencing in BMS-S than luciferase.

In BMS-S3, a similar distribution was observed for the MAR flanked 35S::*GUS* events as was observed in BMS-S, except the mean 35S::*GUS* expression level increased approximately 35-fold (Figure 16B). In contrast to 35S::luciferase, the average level of 35S::*GUS* expression was the same with or without flanking MARs.

In BMS-S4, the *Adh1* 5' MAR still had no effect on *GUS* transgene expression, but there was also no longer as much of a marked bimodal expression distribution as was seen for the previous cell lines with *GUS* and all cell lines with luciferase (Figure 16C). In fact, the distribution of expression for the non-MAR events was no longer significantly different from log-normal (Shapiro-Wilk test indicates a P value of 0.07). In addition to the significant change in the shape of the expression distribution, there was also nearly a 100-fold increase in the average expression level compared to BMS-S3 and nearly a 3,500-fold increase compared to BMS-S. In contrast to the 140-fold increase in *GUS* expression seen by Allen et al. (1996) when comparing non-MAR and MAR-flanked events, I observed a difference of 3,500-fold in *GUS* expression levels between cell lines. The significant increase in the mean level of *GUS* expression over time (i.e. from BMS-S to BMS-S4) is

consistent with the idea that these cell lines exhibited a progressive loss of transgene silencing as they aged (Figure 14).

It was clear that the *Adh1* 5' MAR had a different effect on 35S::*GUS* than 35S::luciferase. Comparing Figure 12A and 16A, the MAR flanked luciferase events have a higher peak for the high expressing events and the MAR flanked *GUS* events have a higher peak for the low-expressing events. Similarly, the *Adh1* 5' MAR increased 35S::luciferase average expression levels by 3.6-fold in BMS-S3, but had no effect on 35S::*GUS* expression in this cell-line (compare Figure 12B with 16B).

The data for each treatment in each cell line is bimodally distributed (except *GUS* in BMS-S4), so it was not possible to compare the data sets by analysis of variance. However, means of non-normal data are normally distributed (Steel and Torrie, 1980). By arbitrarily dividing the data for each cell line into three groups, the mean and standard deviation for each of the three groups of data was calculated. These data are shown in Figure 17 for luciferase and *GUS* treatments, with and without flanking MARs in each cell line. The difference in the means between non-MAR and MAR-flanked events for luciferase in BMS-S indicates that the MAR is able to prevent silencing of many of the events. The mean for the MAR-flanked luciferase events does not change significantly between the different BMS cell lines, but in BMS-S4 there is a significant increase in the mean expression of the non-MAR events. This suggests that in BMS-S4, luciferase was less susceptible to silencing. Similarly, in the *GUS* events the increase in average expression in each sucessive BMS cell line implies that there was successively less silencing in

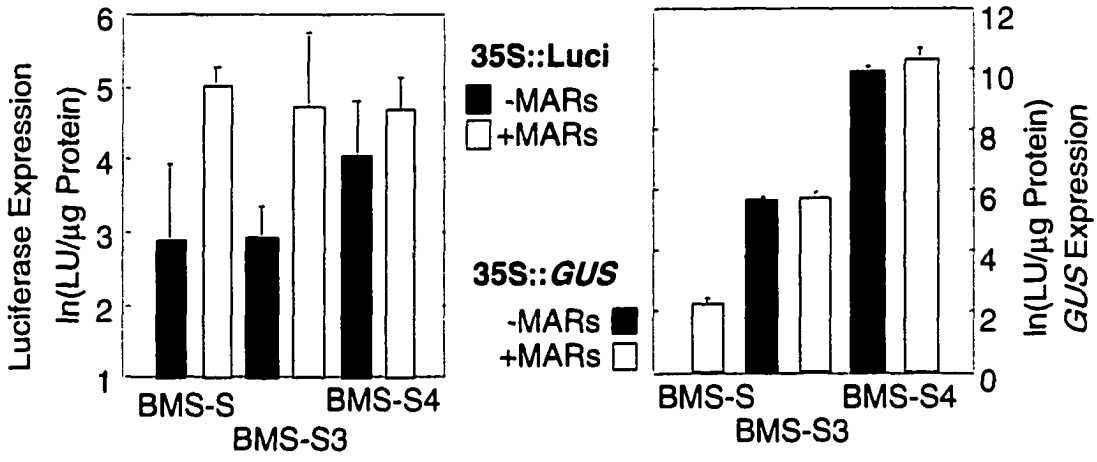


Figure 17. Mean expression level for luciferase and *GUS* in each cell line. Combined results were divided into three random groups. The average of the three groups was graphed along with the standard deviation

BMS-S3 and BMS-S4. In contrast to luciferase, the *Adh1* 5' MAR had no effect on mean *GUS* expression levels in any of the cell-lines.

4.2.4 Transgene copy number can mimic *Adh1* 5' MAR effects on transgene silencing

All of the experiments to this point had been done at a DNA concentration shown previously to give an average of one to two copies per genome (Drummond et al., 1991) and unpublished data, Figure 4). Many studies of MARs flanking transgenes have been done at a much higher copy number (Stief et al., 1989; Phi-Van et al., 1990; Allen et al., 1993; Allen et al., 1996) with some events having greater than 100 transgene copies per genome (Phi-Van et al., 1990; Allen et al., 1993). Allen et al. (1996) suggested that one effect of MARs elements may be to reduce the likelihood of homology-dependent gene silencing, which is a common epigenetic phenomenon seen in transgenic plants (Jorgensen, 1990; Matzke and Matzke, 1995). To determine whether the *Adh1* 5' MAR could also attenuate

the effects of homology-dependent gene silencing, I examined the effects of increasing the DNA dose by 100-fold compared to my standard DNA dose. The average transgene copy number at this DNA dose in BMS ranges from about 10 to 200 (B. Drummond, M. Ross, D. Bond, G. St. Clair and B. Bowen, unpublished). At each DNA dose, equal amounts of 35S::*GUS*, 35S::luciferase, and 35S::*BAR* were introduced on separate vectors in *trans*-. In one set of treatments, all three genes were flanked by the *Adh1* 5' MAR, whereas in the control treatments, none of the genes were flanked by MARs. The results of this experiment are shown in Figure 18.

At the 1x DNA dose, expression level distributions for luciferase were very similar to those seen earlier in BMS-S (Figure 10 and 12), with the larger peak of the bimodal distribution corresponding either to the low expressing events for the non-flanked events or to the high-expressors for the *Adh1* 5' MAR flanked events, respectively (Figure 18A). Surprisingly, the 100x DNA dose also increased the mean 35S::luciferase expression level, and at the high dose, the expression level distributions for MAR-flanked and non-flanked 35S::luciferase showed less of a difference than at the low DNA dose.These data indicate that the net effect of increasing transgene dose in BMS-S3 was similar to the effect that the *Adh1* 5' MAR had on luciferase expression at 1x dose in BMS-S (compare Figure 12A and 18A). A similar type of shift was also seen when comparing 35S::*GUS* without MARs at the 1x verses 100x DNA doses (Figure 18B). These results indicate that increasing transgene copy number decreases silencing of both 35S::luciferase and 35S::*GUS* in BMS-S3, which is contrary to many studies that have reported homology-dependent

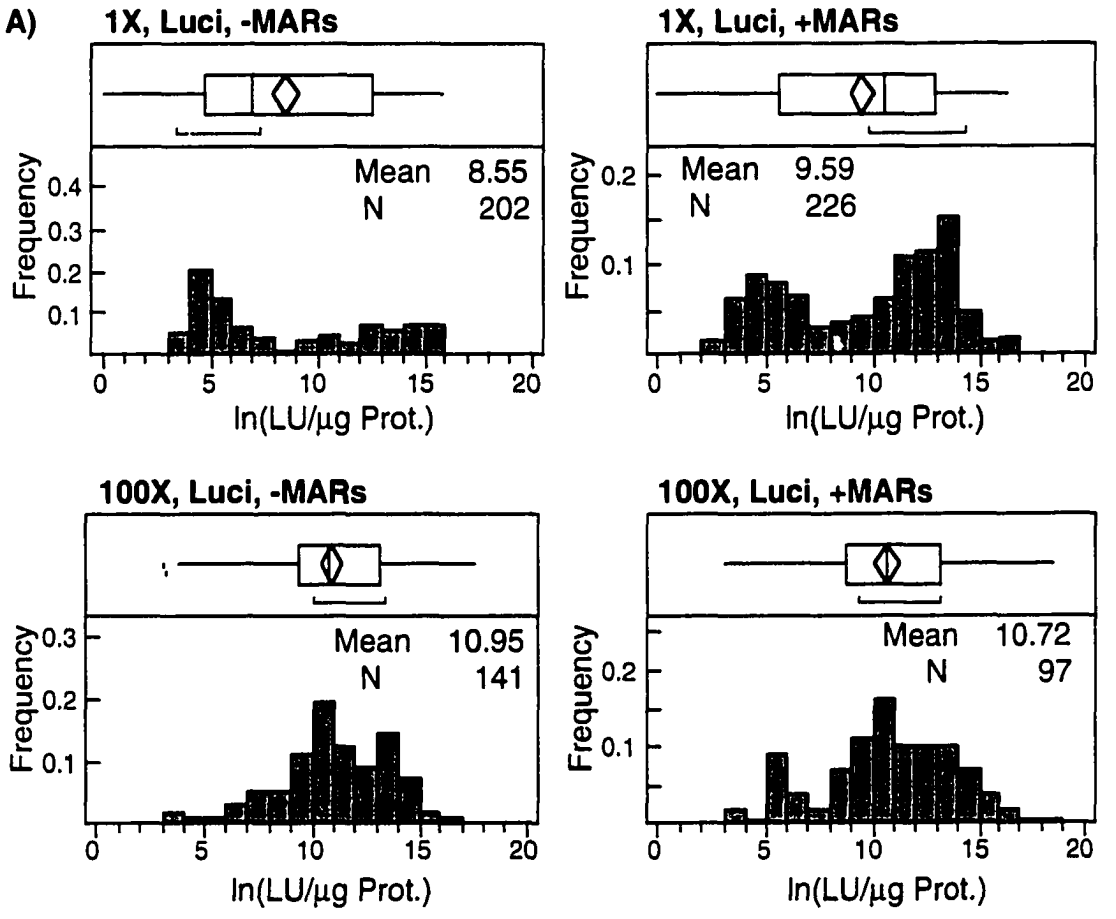


Figure 18. Effect of transgene copy number on 35S::luciferase and 35S::*GUS* expression. 35S::luciferase and *35S::GUS* +/- MARs were transformed into BMS-S3 together in *trans*- at 1x or 100x the normal dose of DNA used for bombardment. The same events were assayed for both luciferase and *GUS* expression. Results from luciferase and *GUS* assays are shown in (A) and (B), respectively.

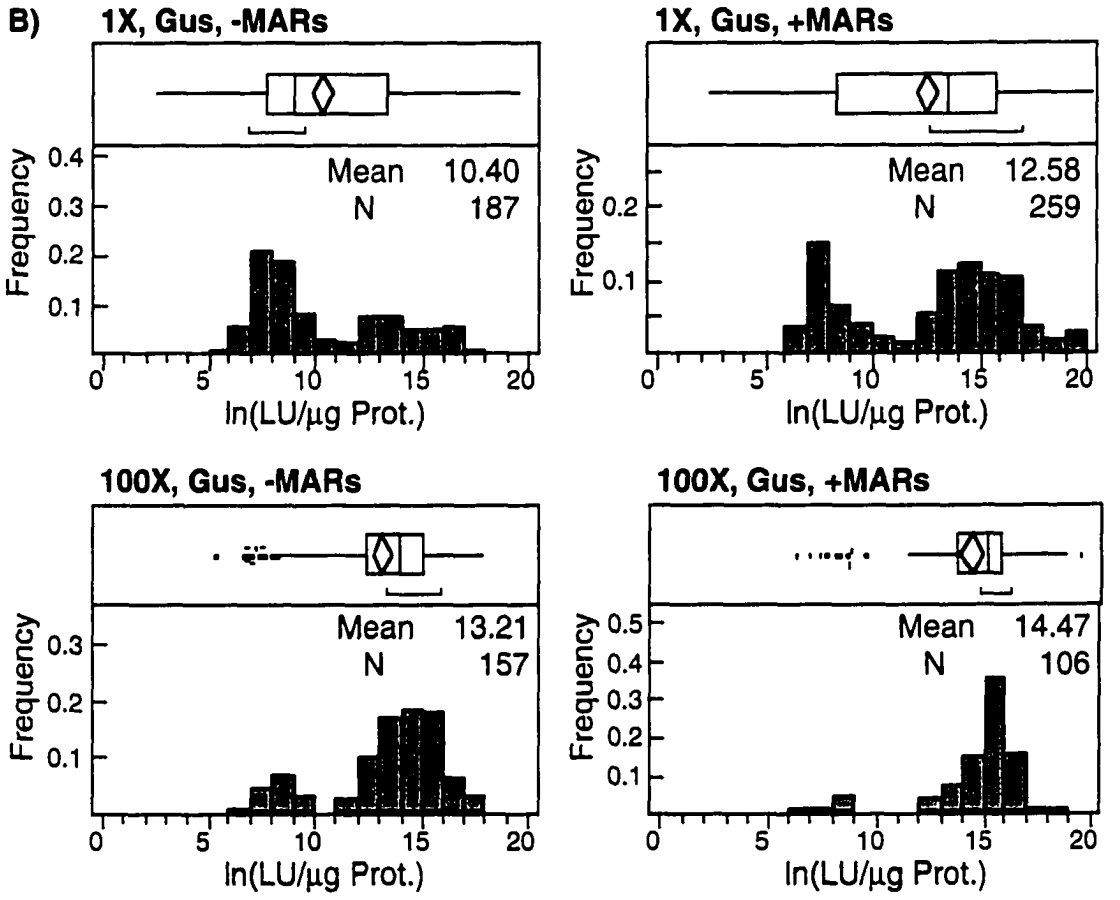


Figure 18. *continued*

silencing associated with multiple copies of a transgene sequence (for reviews see: Jorgensen, 1990; Matzke and Matzke, 1995; Dorer, 1997).

Combining 35S::luciferase and 35S::*GUS* together in the same treatment also had an effect on the MAR by transgene interaction noted earlier in Section 4.2.3. When 35S::*GUS* was transformed into BMS without 35S::luciferase at 1x DNA dose, flanking MARs had no effect on expression (Figure 16). However, when MAR-flanked 35S::*GUS* was introduced together with 35S::luciferase flanked by MARs, the mean level of *GUS* transgene expression was 8.8-fold higher than the control treatment without MARs (Figure 18). In contrast, the effect of introducing MAR-flanked 35S::*GUS* in *trans*- on the increase in mean expression mediated by flanking 35S::luciferase with MARs was negligible by comparison (Compare Figure 12A and 18A). The effect of MAR-flanked 35S::luciferase on MAR-flanked 35S::*GUS* seen here may be mechanistically similar to the synergistic effect of combining multiple MARs described in Section 4.2.1.

4.2.5 Transgene transcriptional states are established at the cellular level

Figure 19 depicts the covariation in 35S::luciferase and 35::*GUS* expression for the four treatments described in Section 4.2.4. The data show that the parameters responsible for establishing and maintaining the transcriptional state of each transgene exhibit a mutual dependence. If each introduced transgene has an independent probability of being turned on or off, then there should be four peaks in each of the graphs shown in Figure 19, that correspond to each of the possible outcomes (i. e. low/low, low/high, high/low, or high/high expression respectively). At the 1x DNA dose, the majority of events without

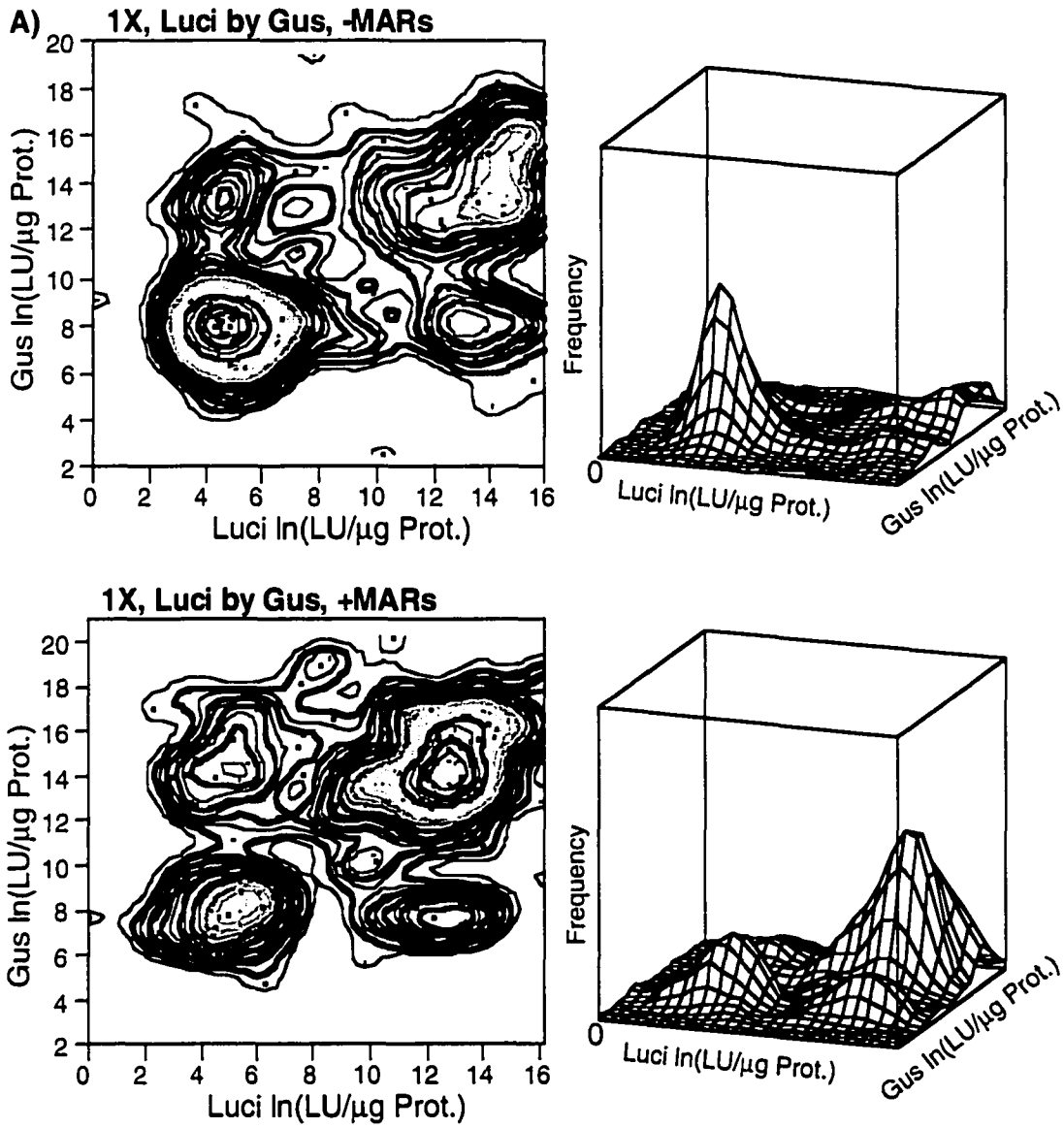


Figure 19. Co-variation in 35S::luciferase and 35S::*GUS* expression +/- MARs at 1x and 100x DNA dose. The same data presented in Figure 18 are graphed as nonparametric bivariate density plots of luciferase vs. *GUS* expression. Contours bound the data points according to their density. Beside each contour graph is a mesh plot diagram of the same data. Data for 1X DNA dose are shown in (A) and 100X DNA dose in (B). These graphs show that at 1x DNA dose, both transgenes tend to express at the same level. At the 100x dose, the transgene copy number masks the effects of the flanking MARs seen at the 1x dose, and the mean level of luciferase low-expressors is raised about 150-fold.

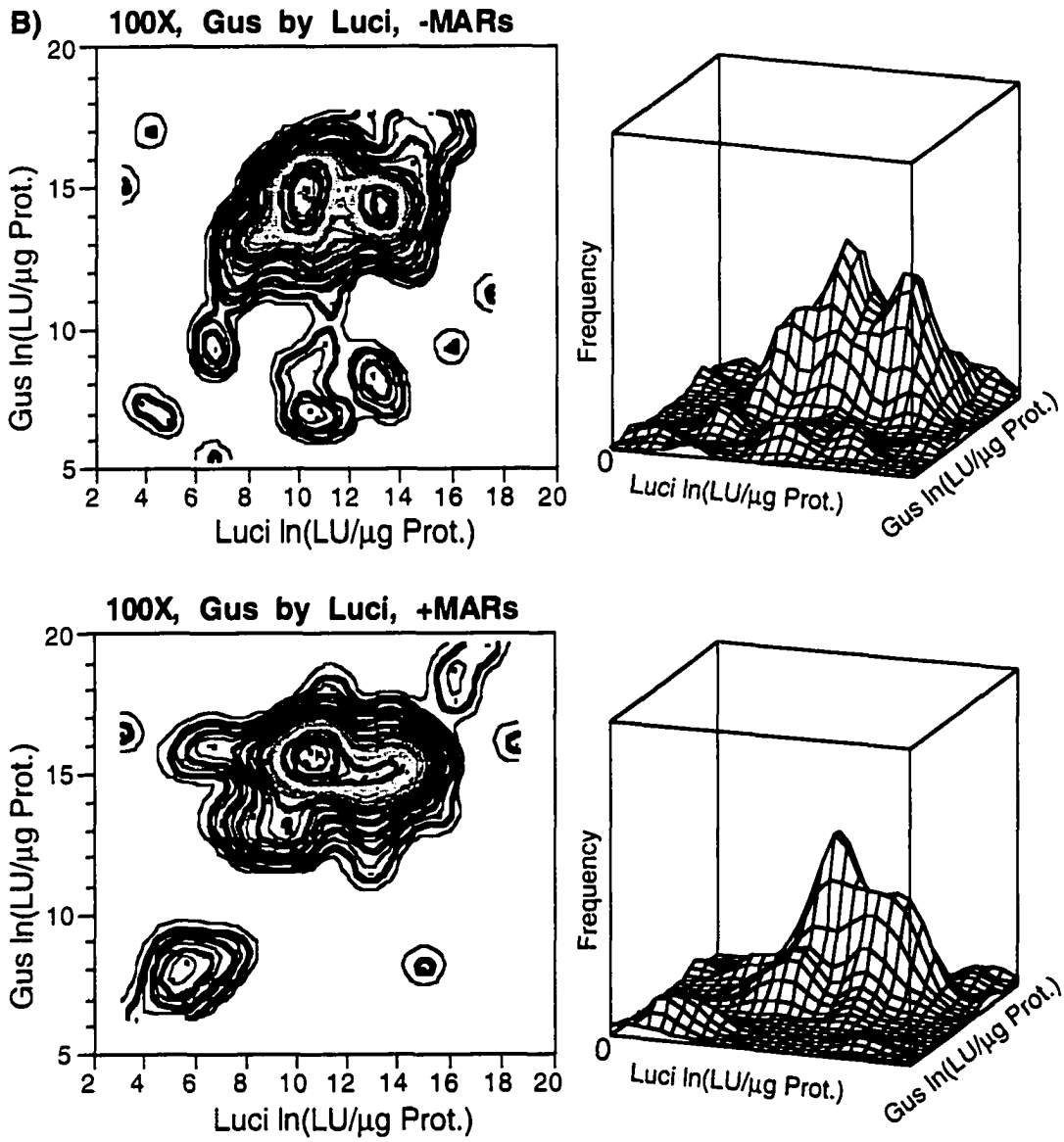


Figure 19. continued

MARs exhibit a low/low pattern of *GUS*/luciferase expression (Figure 19A). When both transgenes are flanked by MARs, the majority of transformants exhibit either a low/low or a high/high pattern of *GUS*/luciferase co-expression, and very few events exhibit high/low or low/high patterns. This indicates that the probability of each transgene being silenced is not independent, but must be determined by the epigenetic state of the cell at the time when transcriptional states are established. Flanking each transgene with MARs dramatically increases the likelihood that an active transcriptional state is established, irrespective of the epigenetic state of the cell. This provides further evidence that MARs prevent transgene silencing by interacting with factors that establish and/or maintain a transcriptionally silenced state. At the 100x dose, the majority of events exhibit high/high or high/low *GUS*/luciferase co-expression, irrespective of flanking MARs (Figure 19B). Interestingly, the average level of luciferase expression in the "low" peak is about 150-fold higher with the 100x dose than with the 1x dose (Compare Figure 19A and 19B). This has a net effect of compressing the bimodal distribution so that it looks almost normal (Figure 18).

4.2.6 *Adh1* 5' MAR affects the maintenance and/or heritability of transgene transcriptional states

English and Baulcombe (1996) reported that silencing of *GUS* was correlated with methylation of sites in the *GUS* gene. To see whether DNA methylation was involved in the silencing of transgenes in BMS, I investigated the effects of S-adenosyl-L-homocysteine (SAH), a demethylating agent reported to have none of the toxic side effects of 5-azacytidine (De Cabo et al., 1994; De Cabo et al., 1995). The data from these experiments were analyzed in two ways. Section 4.2.6.1 summarizes the analysis of pooled data

obtained with events from BMS-S3, S4 and S5, whereas in Section 4.2.6.2, the data from events obtained in each cell-line are analyzed separately.

4.2.6.1 Effects of SAH on gene expression in cell lines -/+ MARs.

Figure 20 shows the effect of SAH treatment over time on the expression level distributions of 35S::luciferase and 35::*GUS* with and without flanking MARs. The main effect of SAH on 35S::luciferase expression is to change the transgene expression level distribution from a bimodal distribution to a more normal type of distribution (Figure 20A). This effect is similar to the effect of increasing copy number in 35S::luciferase events (compare Figure 18A and 20A) and results mainly from a progressive increase in the mean expression level of the low-expressors in response to SAH. After two days on SAH, the difference between MAR-flanked and non-flanked 35S::luciferase expression seen at Day 0 disappears. The effect of SAH on 35S::*GUS* expression in the absence of MARs is similar to that seen for 35S::luciferase (Figure 20B). In contrast to the trend observed with 35S::luciferase, however, the MAR-flanked and non-flanked 35S::*GUS* distributions started out looking more or less identical prior to SAH treatment, but then showed a big difference in response to SAH over time. The mean level of 35S::*GUS* expression without MARs increased much more rapidly over time than the mean level seen among events expressing MAR-flanked 35S::*GUS* (Figure 20B). This effect was mainly caused by the slower rate at which the MAR-flanked events that expressed *GUS* at low levels were derepressed by SAH.

Figure 21 summarizes the different effects of SAH treatment on the mean expression levels of 35S::luciferase and 35S::*GUS* with and without flanking MARs. SAH clearly

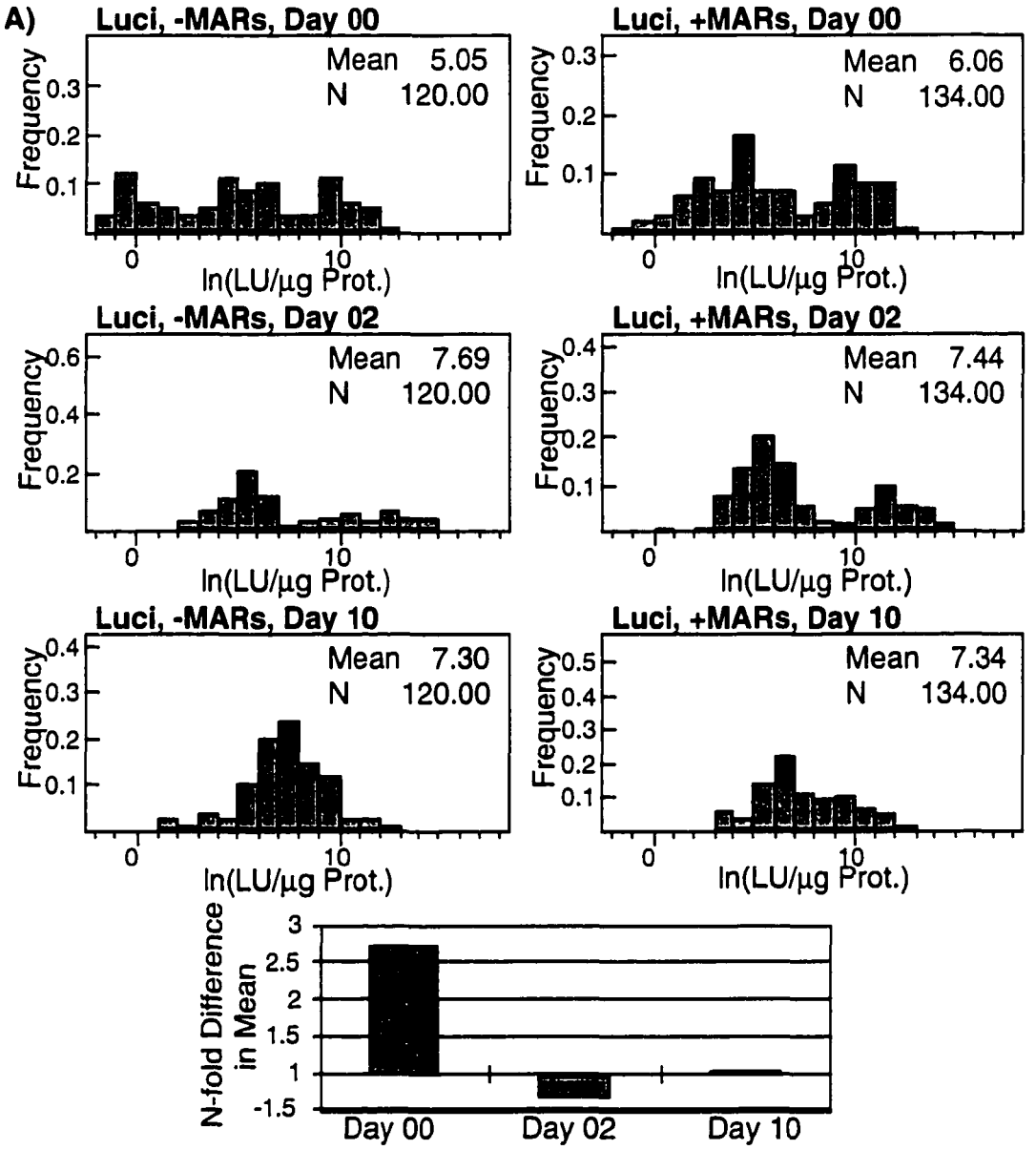
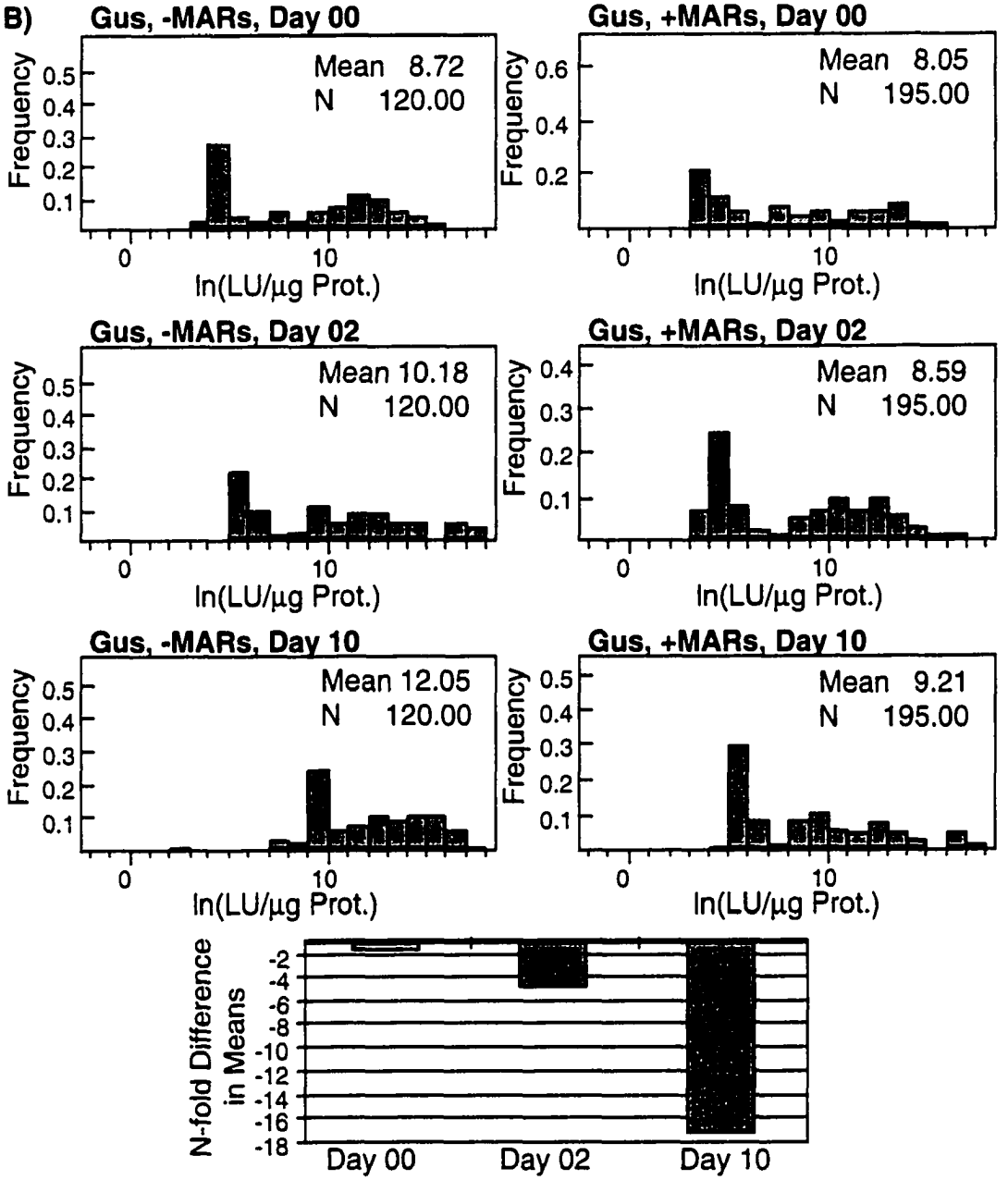


Figure 20. Effect of S-adenosyl-L-homocysteine (SAH) on expression of 35S::luciferase and 35S::*GUS* -/+ MARs. Transformants were assayed at time zero and transferred to solid media containing 150 µM SAH. Samples were taken at 2 days and 10 days and assayed. Distributions shown are from pooled data obtained with transformants derived from BMS-S3, S4 and S5. Luciferase expression levels are shown in (A) and *GUS* data are shown in (B). Below each set of histograms is a graph showing the N-fold difference in average gene expression levels for events + MARs relative to -MARs at Day 0, 2, and 10.



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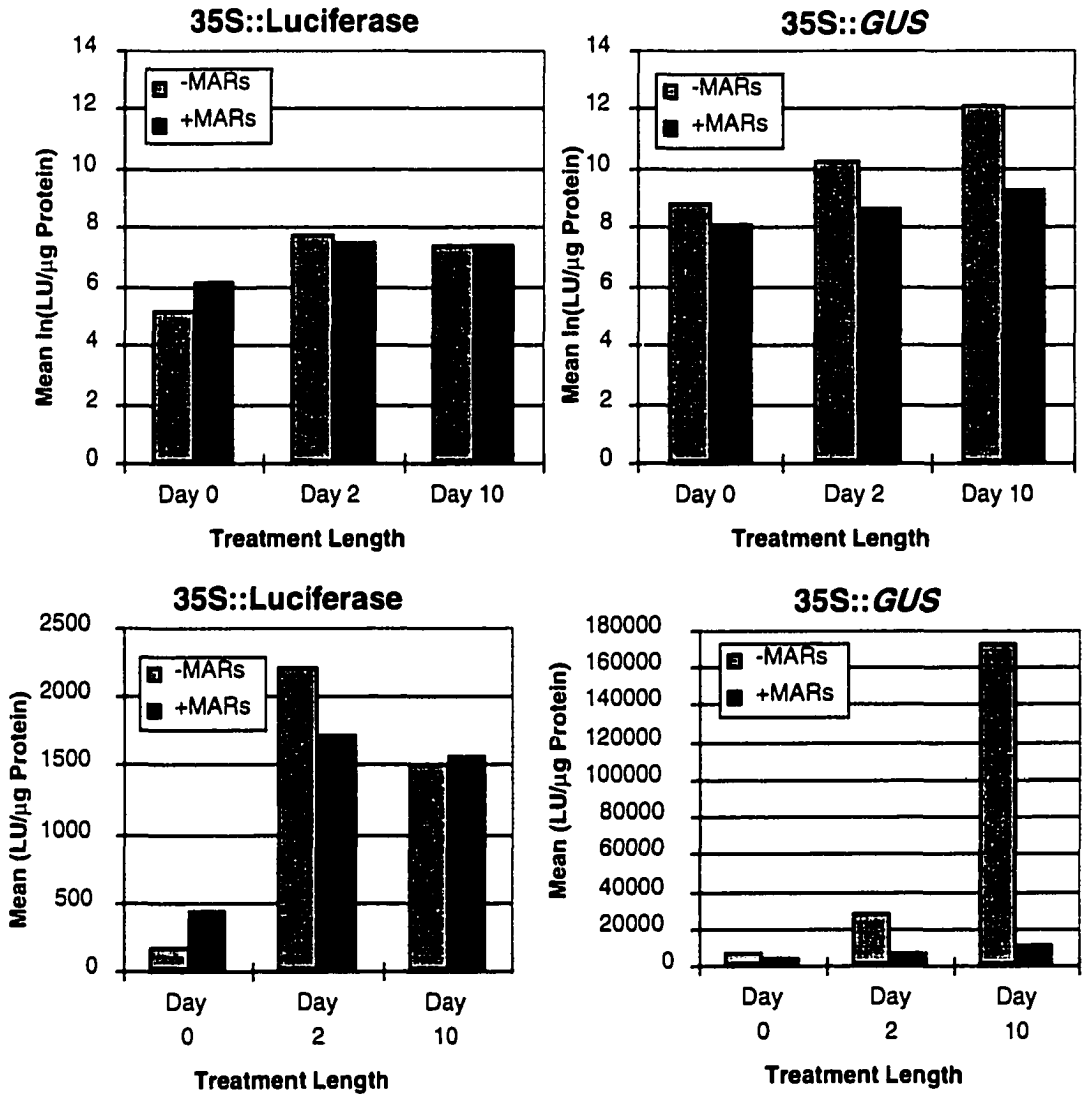


Figure 21. Different effects of SAH on mean 35S::luciferase and 35S::*GUS* expression levels -/+ MARs. Mean levels of expression are graphed as a function of the length of SAH treatment. In the upper two graphs, means are graphed on a logarithmic scale, whereas in the lower two graphs, means are plotted on a linear scale.

derepresses silenced copies of both 35S::luciferase and 35S::*GUS*, and 35S::luciferase was derepressed by SAH at a much faster rate than 35S::*GUS*. However, silenced copies of 35S::*GUS* flanked by the *Adh1* 5' MAR are derepressed at a significantly lower rate than silent copies of non-flanked 35S::*GUS*, an effect which is not as clearly seen for 35S::luciferase because of the difference between MAR-flanked and non-flanked 35S::luciferase level prior to SAH treatment. The data reflect specific transgene by SAH and MAR by transgene by SAH interactions, but demonstrate that the *Adh1* 5' MAR can have an effect on the maintenance and/or heritability of stable transcriptional states in response to perturbations such as SAH treatment. Thus, the same MAR sequence can affect both establishment and maintenance or heritability of stable transcription states *in vivo*.

4.2.6.2 Analysis of SAH effects on transformants obtained with BMS-S3, S4 and S5

Figure 22 shows the results of treating different transformants with SAH when the data are broken out according to cell line of origin. Interpretations of the data are complicated by the small sample sizes for each cell line and by changes in the cell lines over time. Much of the variation in behavior among different cell-lines is apparent in the summary graphs of the N-fold differences in mean expression levels over time below each of the summary sets of histograms in Figure 22A-F. The *GUS* responses in BMS-S3 and the luciferase responses in BMS-S4 reflect the responses seen in the pooled data the closest (Figure 20). Figure 23 shows that, in general, the *Adh1* 5' MAR flanked transgenes (i.e. both 35S::*GUS* and 35S::luciferase) were less likely to be derepressed by the demethylating agent than those without MARs, although the results varied from cell line to cell line,

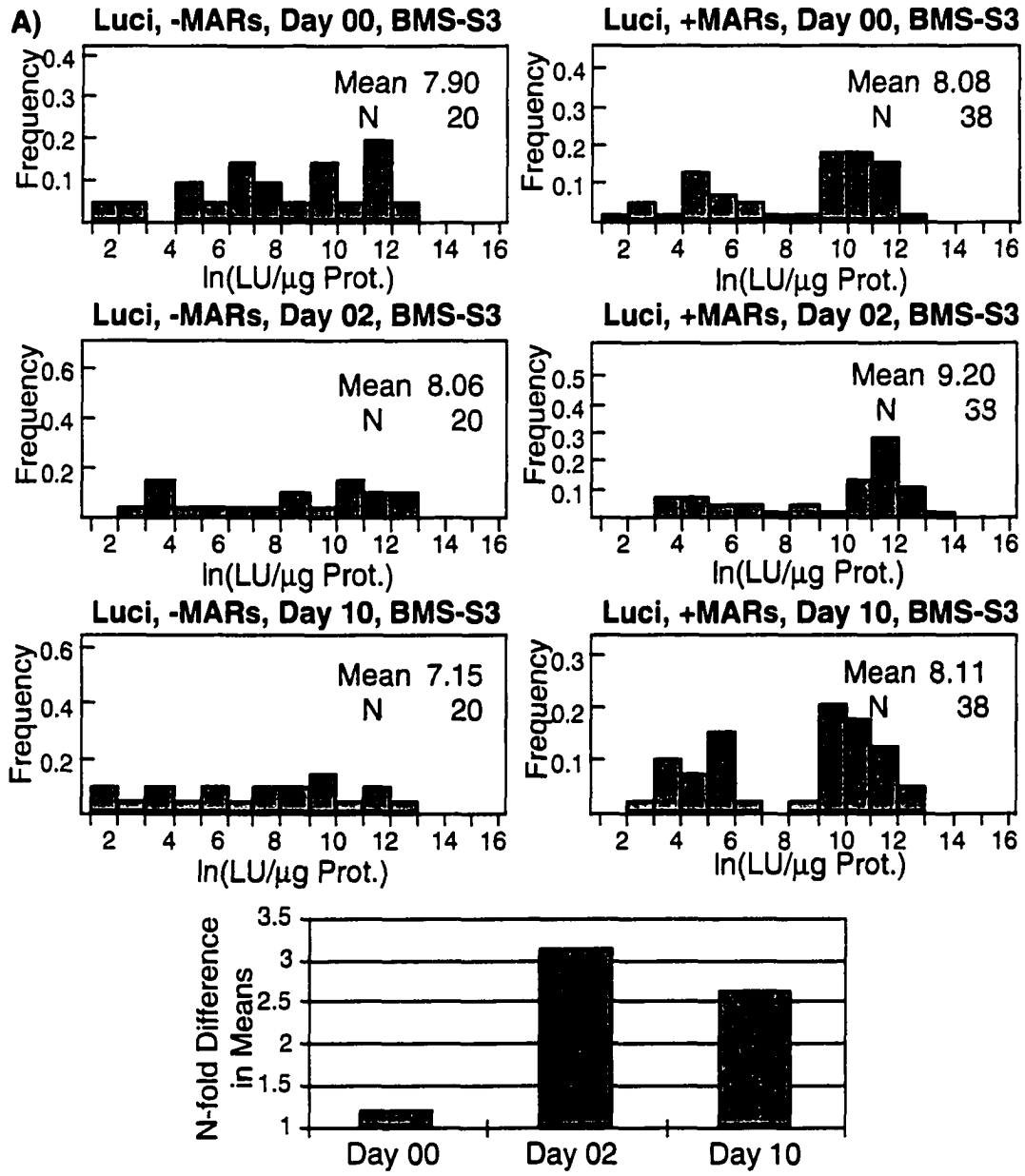
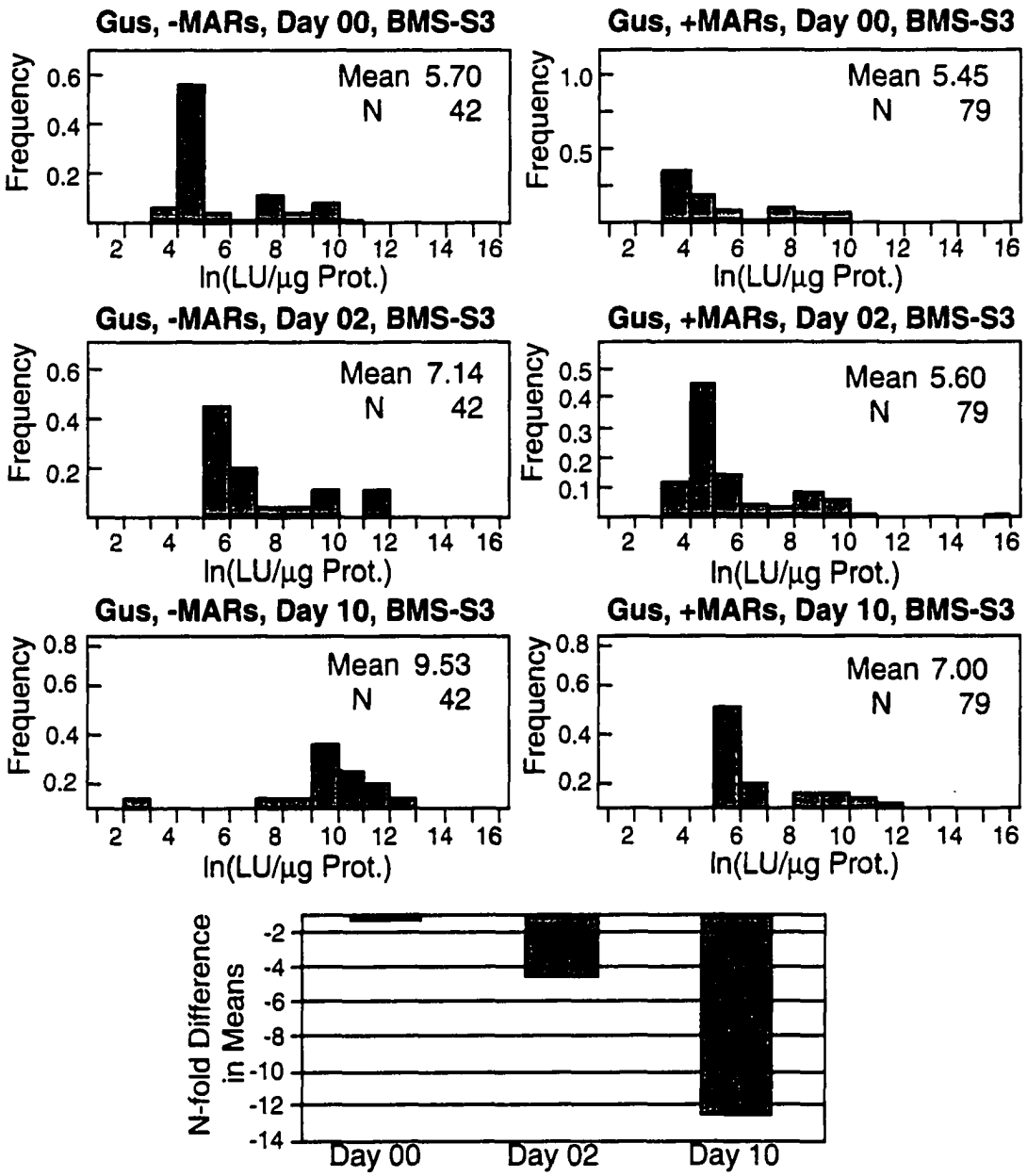


Figure 22. Histograms of SAH results separated by cell line. 35S::luciferase in BMS-S3 (A). *35S::GUS* in BMS-S3 (B). (C) 35S::luciferase events in BMS-S4. D) *35S::GUS* events in BMS-S4. E) 35S::luciferase events in BMS-S5. F) *35S::GUS* events in BMS-S5. Below the histograms is a N-fold difference graph of the means for the three treatment lengths.



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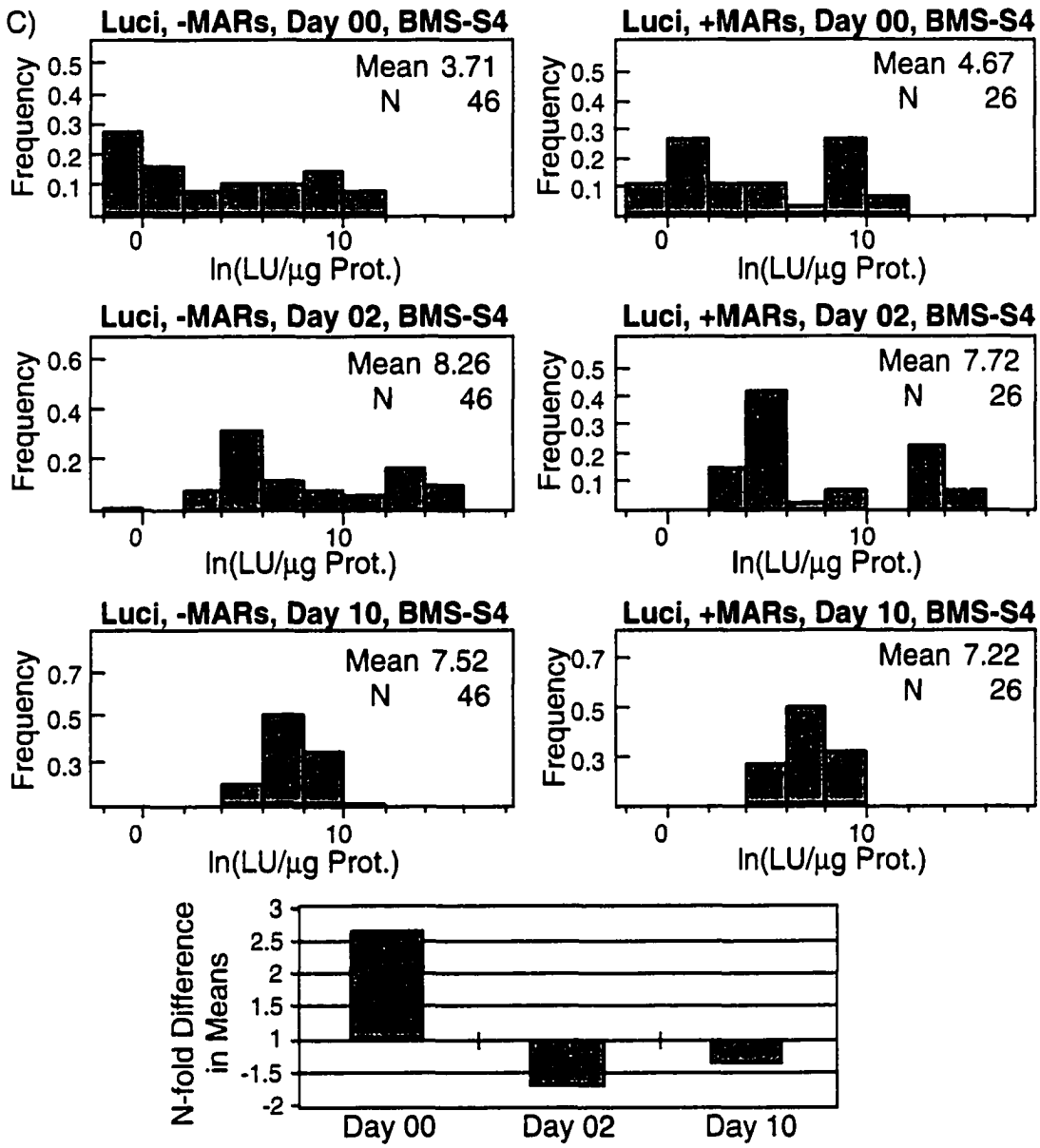

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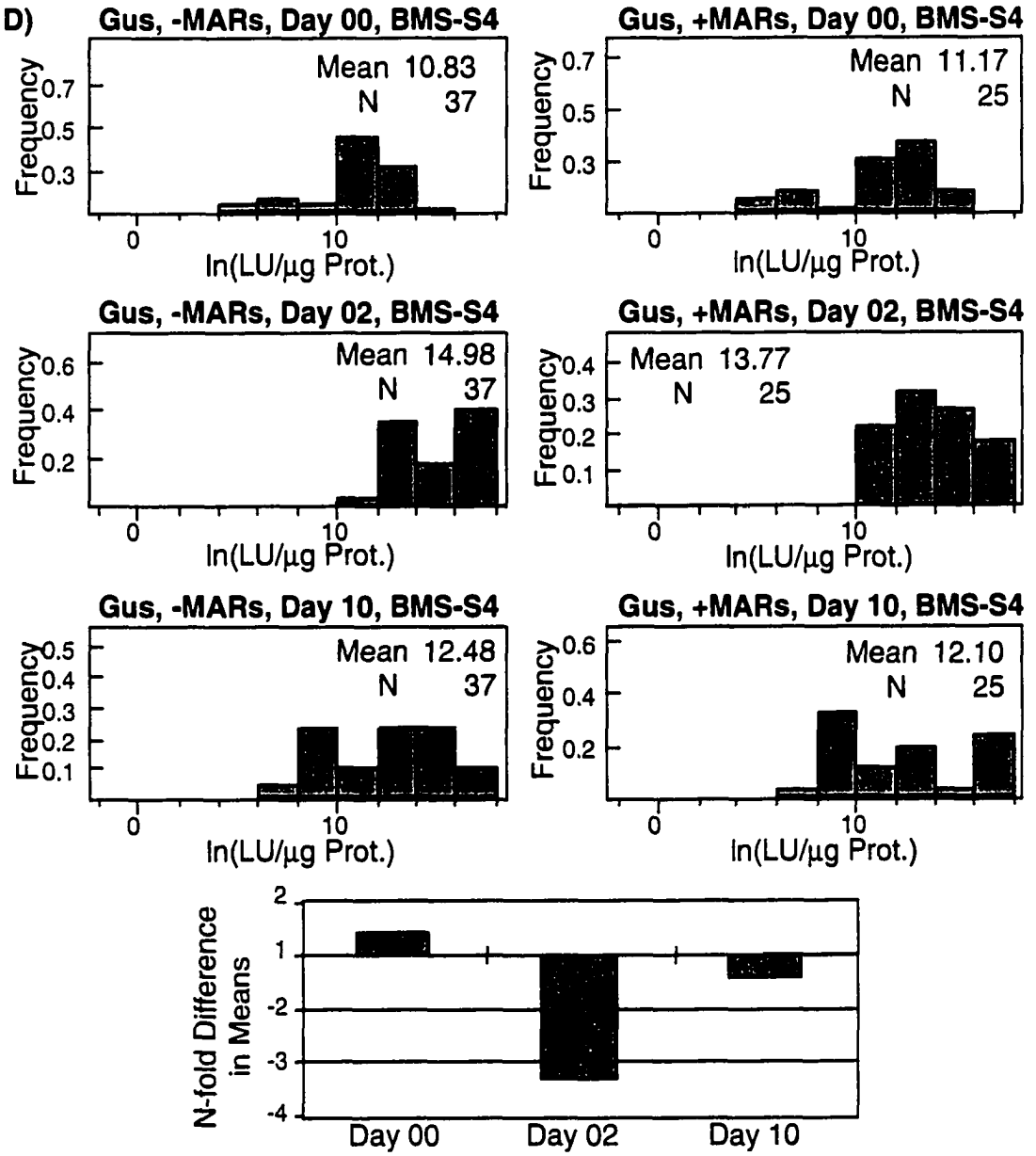

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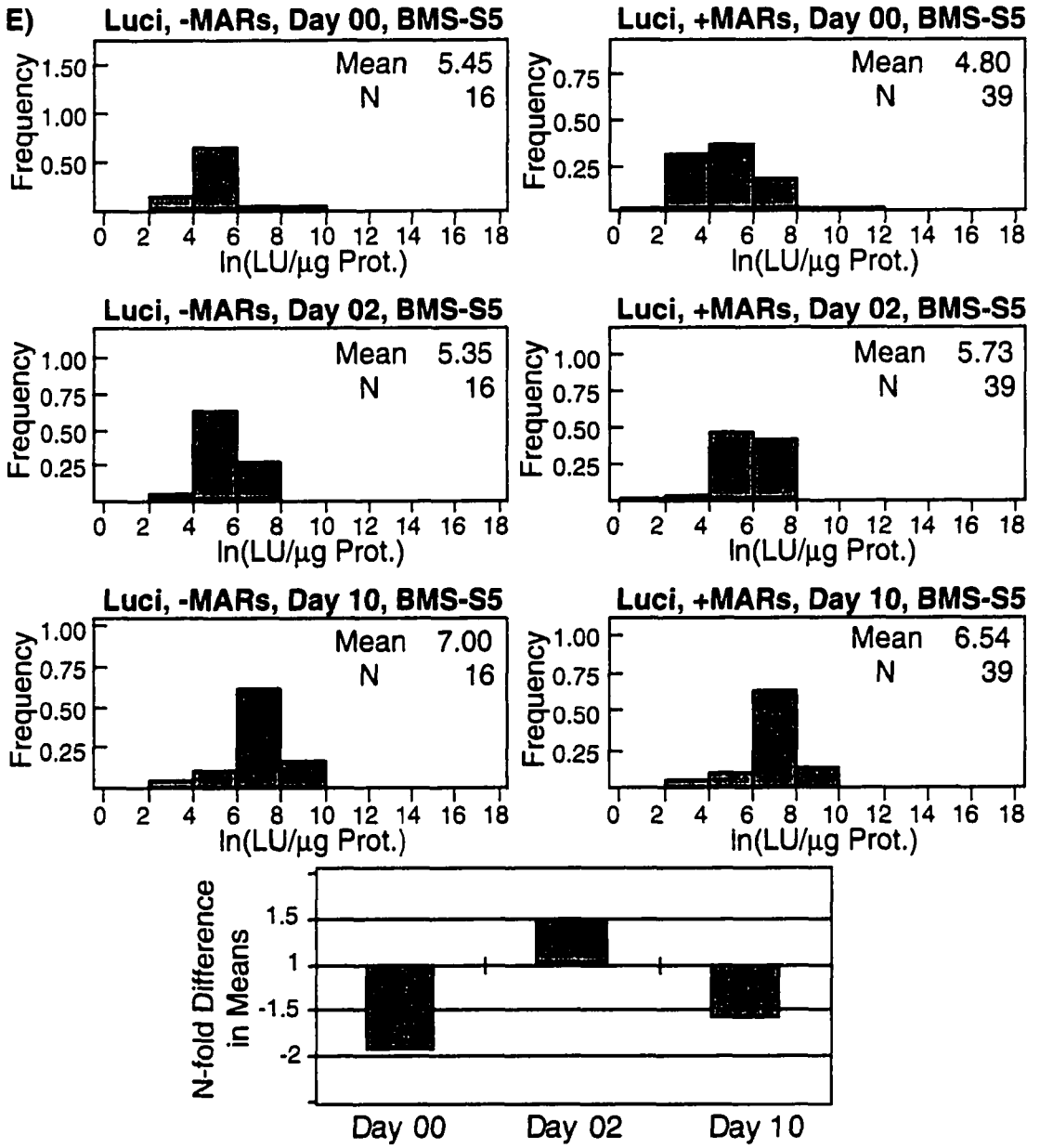

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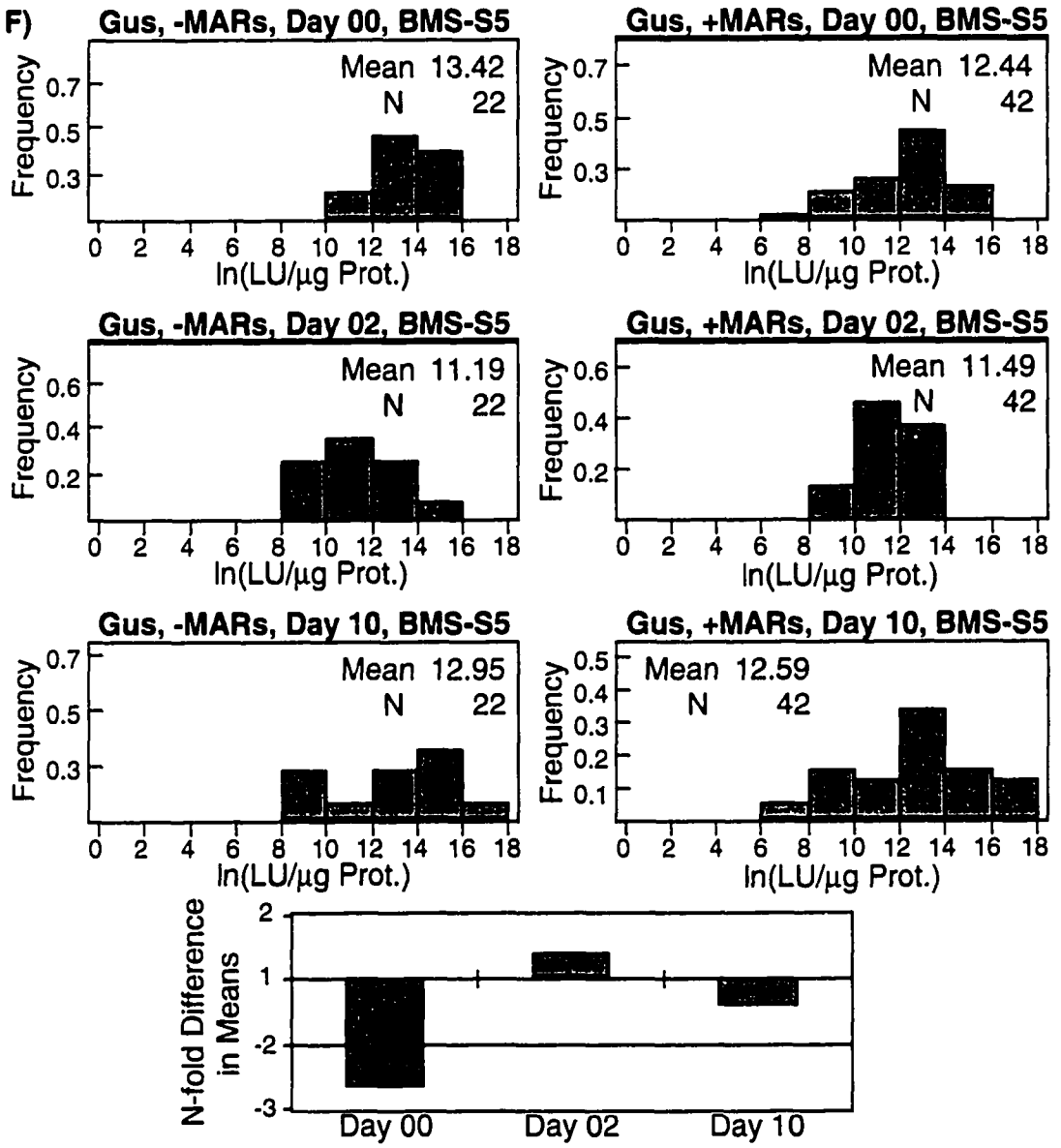

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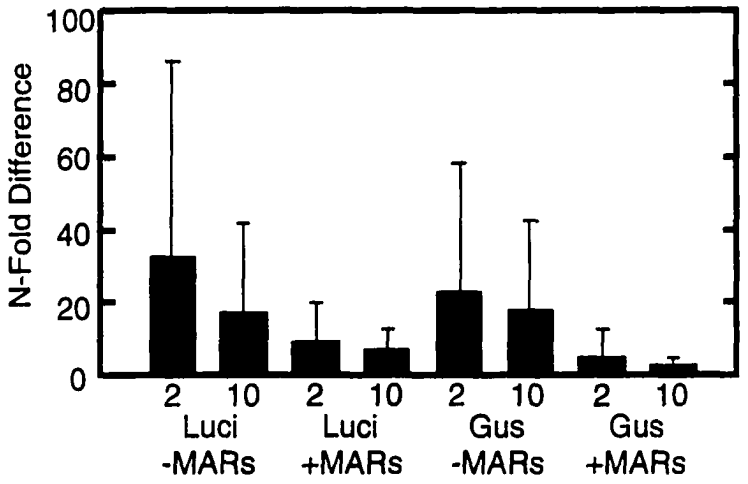

Figure 23. N-fold difference in SAH treated events. Events from each of the above treatments were treated with SAH for 48 hours (2 days) and assayed. Expression levels were compared to the expression level before treatment. Randomly chosen events (n=16-79) for each treatment from 3 different experiments in three different cell lines were grouped.

resulting in large error-bars. This reflects the slower rates of derepression seen in the first two days of SAH treatment for the MAR-flanked events in the pooled data (Figure 21).

Many studies have shown that treatment with the histone deacetylase inhibitor trichostatin A (TSA) can also activate expression of silenced genes (Yoshida et al., 1995; Chen et al., 1997). To determine if histone acetylation could relieve silencing, I treated both 35S::luciferase and 35S::*GUS* events in BMS-S5 with TSA. The results are shown in Figure 24. TSA appeared to have no ability to derepress either GUS or luciferase in this cell line, but this could be because the level of transgene silencing had already been lowered to such an extent to nullify any effects that might have been observed.

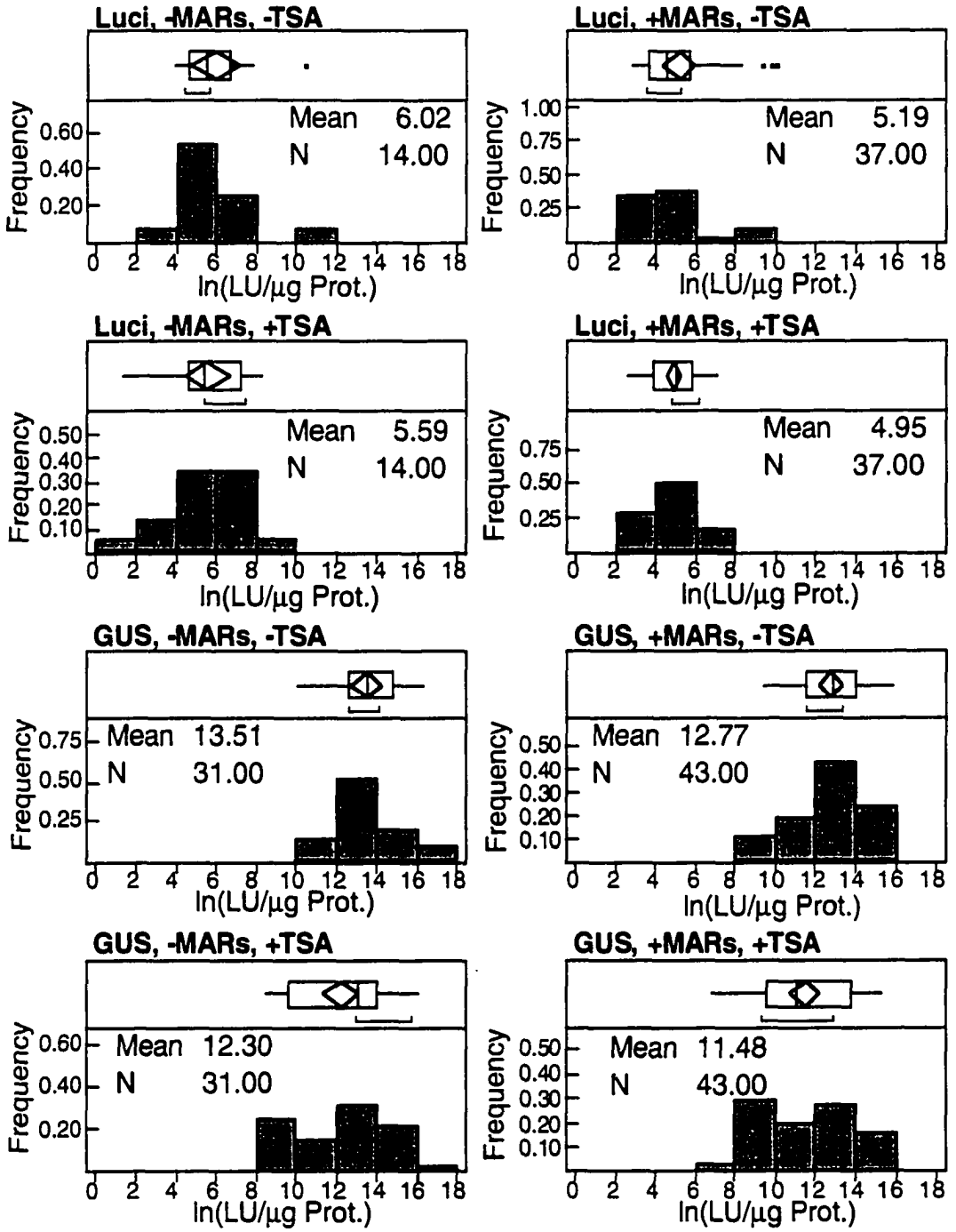

Figure 24. Effects of Trichostatin A (TSA) treatment on 35S::luciferase and 35S::*GUS* expression. Events from BMS-S5 were treated with TSA for 24 hrs. and assayed.

4.2.7 ARS1 effects on transgene expression also change in older BMS cells

The 2 x 2 experiment design employed in section 4.2.1 was also used to investigate the effects of flanking one or both transgenes with ARS1. In contrast to the results obtained with the *Adh1* 5' MAR, the effect of flanking one transgene with ARS1 on expression of an unflanked gene was strikingly different, at least in BMS-S. Figure 25 shows the results. As mentioned earlier, flanking both *BAR* and luciferase with ARS1 (treatment 4) increased the average expression level over that of the control (treatment 1) where no ARS1 sequences were used. In contrast, when only one of the transgenes was flanked by ARS1, expression of the non-flanked transgene was apparently suppressed. For example, when *BAR* flanked by ARS1 was introduced with 35S::luciferase (treatment 2), the average luciferase expression level was near zero for all events analyzed. When 35S::*BAR* was introduced with ARS1-flanked 35S::luciferase (treatment 3) no events were recovered, indicating that the selectable marker must not have been expressed at a high enough level to confer resistance to the herbicide used for selection. This experiment was repeated twice in BMS-S with the same results.

Transgenes introduced on separate vectors typically co-integrate together at one or few integration sites (Czernilofsky et al., 1986; Huang and Dennis, 1989). My working model for the effects of ARS1 seen in Figure 25 invokes the directionality that has been associated with insulator and/or silencer elements in yeast and *Drosophila* (Geyer and Corces, 1992; Cai and Levine, 1995). In this case, ARS1 may prevent silencing of the transgene it flanks, but may actively promote silencing of non-MAR flanked DNA outside of the ARS1-bounded loop. To test this model, I designed and constructed vectors with the

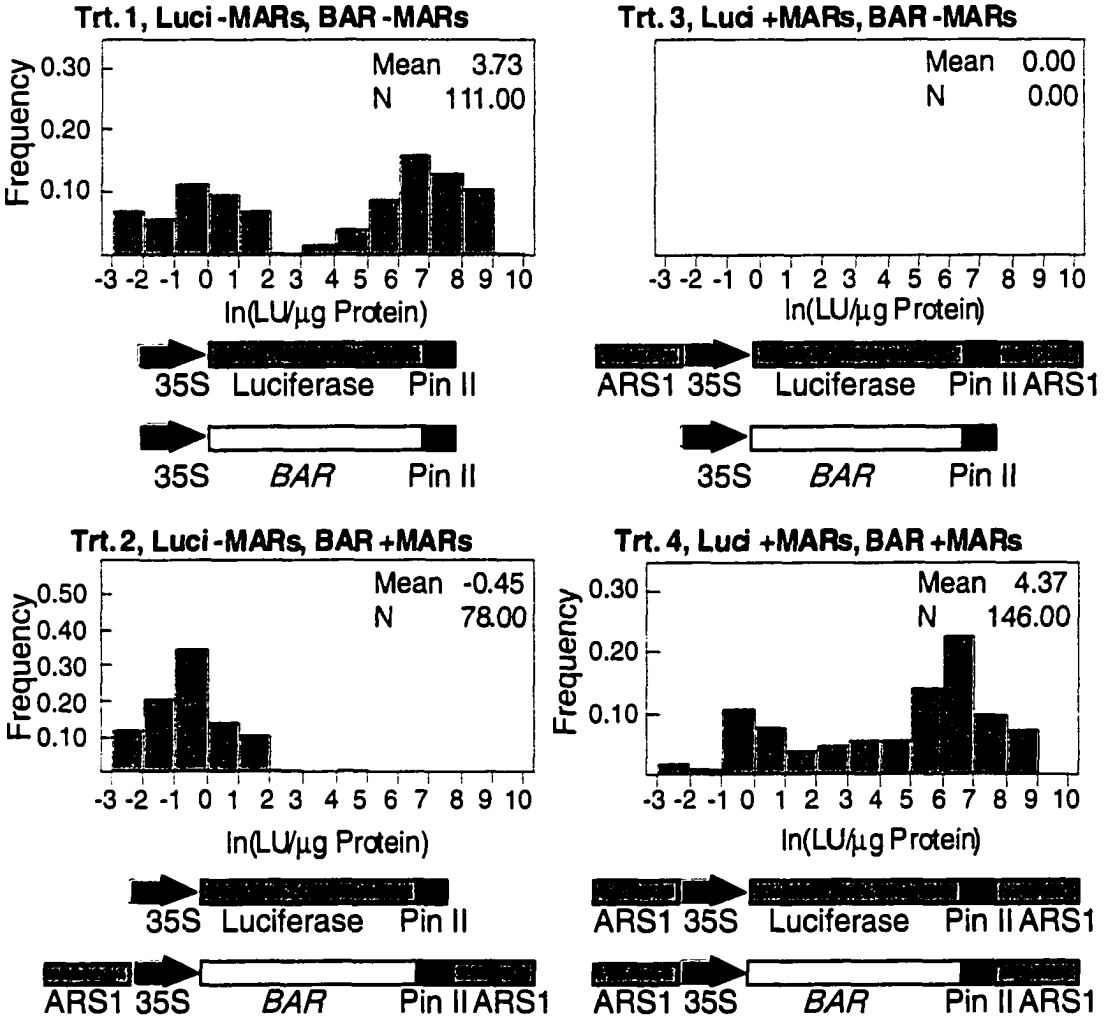

Figure 25. Histograms of ARS1 data. The reporter luciferase or the selectable marker *BAR* were alternately flanked with ARS1 in treatments 2 and 3. In treatment 1 neither of the transgenes were flanked and in treatment 4 both were flanked. Diagrams of the plasmids are below each graph. All treatments were done in BMS-S. Representative data from one of two identical experiments are shown.

selectable marker and reporter genes in *cis*-, where one or the other of the genes was flanked with ARS1. However, by the time I was ready to test these vectors, I was using BMS-S5 for transformation, which as shown earlier, exhibited significantly reduced levels of transgene silencing analogous to those seen in BMS-S4. Figure 26 compares the results of the 2 x 2 experimental design in BMS-S5 using the *cis*- and *trans*- vector combinations. In BMS-S5, the *trans*-combinations (treatments 2 and 3) did not have the same effect as they did in BMS-S and there was essentially no difference between the *cis*- and *trans*-treatments (Figure 26). Thus, I was unable to determine if my model was correct because of the altered responses in BMS-S and BMS-S5. However, the differences seen between BMS-S and BMS-S5 are entirely consistent with the loss of transgene silencing inferred from experiments with the *Adh1* 5' MAR.

4.2.8 Decreases in end-joining parallel loss of transgene silencing in aging BMS cells

In stable transformants, transgenes integrate through the non-homologous double-stranded break repair (DSBR) pathway. Non-homologous DSBR is the predominant pathway for repairing double-stranded breaks in multicellular eukaryotes (e.g. humans, maize) (Fishel et al., 1991; Daza et al., 1996; Shalev and Levy, 1997). In yeast, the predominant pathway for DSBR is homologous recombination involving Rad51/52 mediated exchange. When the Rad51/52 dependent pathway is inactivated in yeast, however, DSBR occurs predominantly via an end-joining process. This involves a heterodimeric protein similar to the Ku antigen that binds dsDNA breaks in higher eukaryotic cells. One of the subunits of this complex, Hdfp1, has recently been shown to interact with silent information regulator

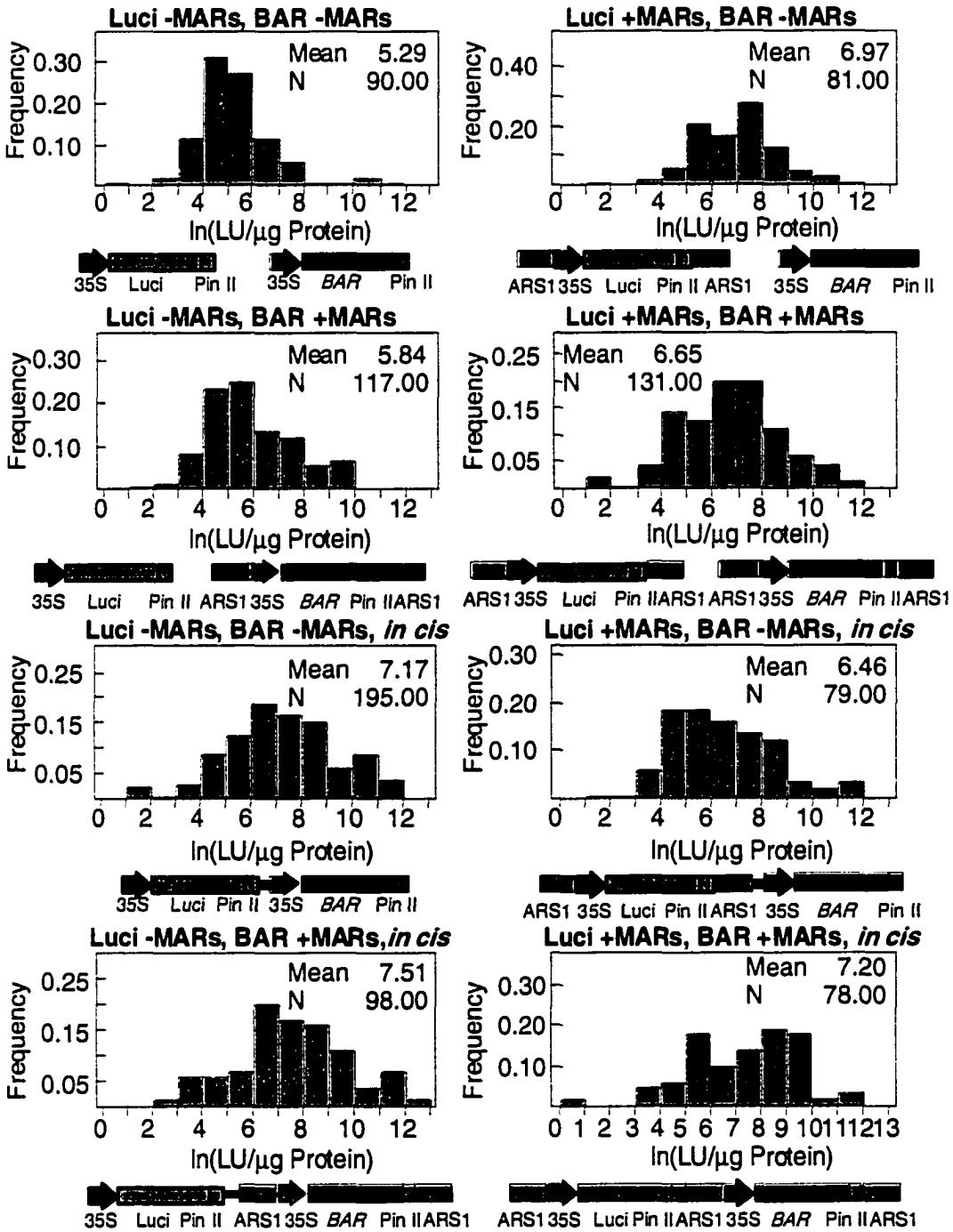

Figure 26. Histograms of ARS1 data in BMS-S5. Treatments are essentially the same as for figure 12, except in the second set of four treatments the reporter and selectable marker were linked together on the same plasmid.

(SIR) proteins that are required for gene silencing in yeast (Tsukamoto et al., 1997). This suggests that for end-joining to occur, the chromatin must be in a heterochromatic state (Lustig, 1998). Besides promoting the loss of gene silencing and reducing the frequency of end-joining, mutations in *sir2*, *sir3*, and *sir4* also decrease the lifespan of the cell (Kennedy et al., 1995; Kim et al., 1996; Smeal et al., 1996; Austriaco and Guarente, 1997; Kennedy et al., 1997). In fact, loss of SIR function and loss of silencing are key features associated with the onset of aging in yeast (Lustig, 1998).

Through the course of my dissertation work, I observed progressive loss of transgene silencing the longer the BMS cells were maintained (Section 4.2.2). Also, because MAR effects on transgene expression are epigenetic in nature, it is likely that *trans*-acting silencing factors could be involved. Could SIR orthologs present in maize cells be responsible? To investigate this idea further, I calculated the number of transformants I obtained per bombardment for each cell line. Because SIR proteins are involved in end-joining, the transformation efficiency should be a good indicator of the level of these proteins present in the cells. Figure 27 shows that the frequency of end-joining decreased from one BMS subculture to the next.

4.2.9 Discussion

In this section, I have demonstrated that MARs can influence the establishment and maintenance or heritability of transgene transcription states in BMS cells. I also showed that the degree of transgene silencing in BMS decreased progressively over a period of five years. This correlated with progressive loss of the protective effect of MARs against

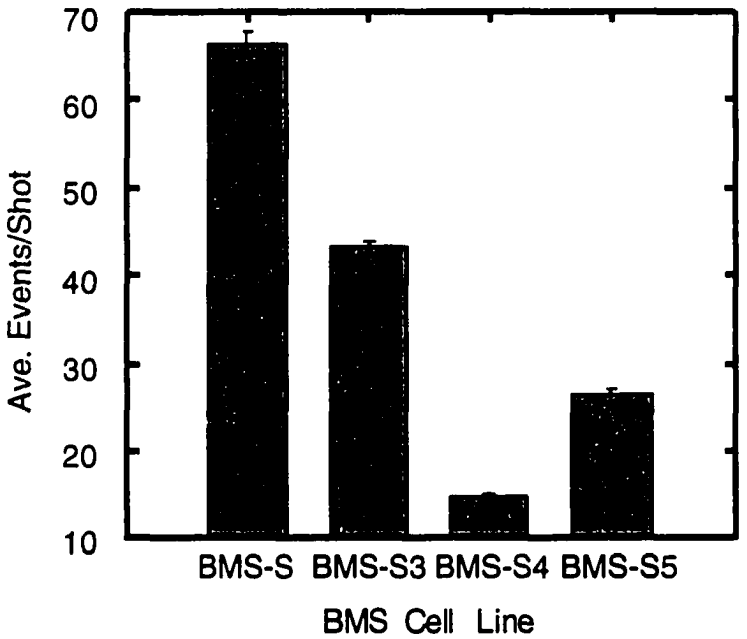

Figure 27. Average number of transformants obtained per bombardment for each cell line. Average number of transformants per bombardment was calculated for all experiments for each cell line. As the cell line ages transformation efficiency decreases. Error bars represent standard error.

transgene silencing, suggesting that *trans*-acting factors responsible for both effects might have been gradually lost from each BMS line over time. My findings in BMS are very similar to observations made in aging yeast cells, where SIR proteins that are responsible for establishment and heritability of gene silencing are lost or rendered functionally inactive over time. In yeast, SIR proteins also interact with the machinery responsible for non-homologous recombination (i.e. end-joining), and loss of SIR function is associated with reduced end-joining. Similarly in BMS cultures, as transgene silencing was lost, the levels of end-joining (as measured by the frequency of stable transformation) also decreased. Thus, factors involved in cellular aging, transgene silencing, end-joining, and the *in vivo*

effects of matrix attachment regions in higher eukaryotic cells such as BMS may be interrelated, much as they are in yeast. Unfortunately, since the effects in BMS were observed over a 5-year period, when the culture had already been maintained for approximately 20 years, it would be difficult to repeat these experiments. To test whether loss of transgene silencing and the protective effects of MARs was truly associated with plant cell age, a system would need to be developed where the number of cell divisions could be tracked (Kennedy et al., 1995) , and, preferably, an enrichment procedure for old cells could be devised (Smeal et al., 1996). This type of system might be able to determine if the changes I saw in BMS are common symptoms of aging in plant cells, or if other factors that changed during the five year period of my dissertation were responcible.

The *Adh1* 5' MAR effects on transgene expression are epigenetic in nature and were not stable over time, because they depend on how long the cell line was maintained in culture and its capacity for silencing. I have been able to separate four variables that interact to influence expression of transgenes in BMS cells: MAR elements, transgene coding sequence, cell-line, and transgene copy number. I also found that differeent MAR by transgene combinations affected the response to SAH treatment. Table 5 summarizes some of the interactions between these factors that I observed.

MAR by transgene interaction. Flanking luciferase with *Adh1* 5' MAR prevented silencing of luciferase in BMS-S and BMS-S3 and increased the average expression level. This result is consistent with the most commonly observed effect of MARs in plant transgene expression studies (Breyne et al., 1992; Allen et al., 1993; Schöffl et al., 1993; Mlynarova et al., 1994; van der Geest et al., 1994; Mlynarova et al., 1995; Allen et al., 1996;

Table 5. Summary of interactions affecting transgene expression in BMS.

Interactions	MAR	Transgene	Cell-Line	Copy Number	SAH Response
1	X	X			
2	X		X		
3	X	X	X		
4	X	X		X	
5	X	X			X
6		X			X

van der Geest and Hall, 1997). However, flanking 35S::*GUS* with *Adh1* 5' MAR had no effect on *GUS* expression levels in BMS-S3. My data suggest that silencing of *GUS* and luciferase may involve different mechanisms, because in one case (i.e. 35S::luciferase) silencing is overcome by flanking MARs, whereas in the other (i.e. 35S::*GUS*), MARs have little effect. However, as MAR copy number is increased, the likelihood of escaping from silencing is increased, even for 35S::*GUS* (Section 4.2.4), suggesting that the molecular basis for MAR by transgene interactions may involve competition between silencing and anti-silencing factors *in vivo*. Studies have shown that *GUS* is susceptible to silencing and that silencing is associated with and maintained by methylation (Baulcombe and English, 1996; English et al., 1996). In BMS, luciferase could be silenced by a different mechanism (e.g. by changes in histone acetylation). Our data indicate that silenced transgenes may exhibit different rates of derepression in the presence of SAH, depending on the sequence of the transgene itself and whether it is flanked by MARs. This derepression could be the result of demethylation caused by the SAH treatment (if SAH action in BMS is the same as in mammalian cells (De Cabo et al., 1994; De Cabo et al., 1995)). Different rates of derepression may reflect either differentiated access to demethylation enzymes *in*

vivo or differential loss of silencing factors following inhibition of maintenance methylation by SAH.

MAR by cell line interactions. The *Adh1* 5' MAR had the greatest effect on luciferase expression in BMS-S(Figure 12A and 13). In BMS-S3 and BMS-S4, the difference between luciferase with or without flanking MARs decreased. MAR by cell-type interactions have been reported previously in a mouse system. There, a HSP70.1 promoter driven reporter was stimulated by flanking MARs (referred to as SARs in their report) in mouse embryos but not in differentiated tissues (Thompson et al., 1994). In contrast to the mouse study, BMS cell lines which showed different MAR effects did not differentiate. The differences observed over time could reflect either the age of the cell culture of other changes in cellular metablolism that occured during the 5-year period of study.

The *Adh1* 5' MAR prevented silencing of luciferase, but not *GUS* in BMS-S3. However, silencing was relieved in BMS-S4 which masked the MAR effect on luciferase expression. This is reflected by the increase in average gene expression for *GUS* treatments with and without MARs in BMS-S3 and BMS-S4. In both treatments, *GUS* expression increased over 100-fold from BMS-S3 to BMS-S4. The average expression level among non-flanked luciferase events also increased, but not to the extent of the *GUS* events. However, the average expression of MAR flanked luciferase events did not increase between the cell lines.

This loss of silencing in BMS is an epigenetic event that could be caused by the length of time in culture. In yeast, the SIR proteins involved in derepression of telomeric silencing are also involved in non-homologous double strand DNA break repair. Homology

between yeast proteins involved in this pathway and mammalian proteins indicate that these proteins may be common to all eukaryotes (Maize also has Ku homologs: B. Bowen and P. Mahajan, personal communication). A good measure of the efficiency of non-homologous recombination is transformation efficiency, since transgene integration requires the non-homologous double strand DNA break repair pathway for integration (Smih et al., 1995; Dellaire and Chartrand, 1998). The good correlation between the progressive decrease in silencing and transformation efficiency throughout the course of my experiments closely parallels the situation found for aging yeast cells. Thus, many of the MAR by cell-line interactions I observed most likely are the result of BMS showing changes over time, similar to aging in yeast.

The unique effect of ARS1 in BMS-S also changed between BMS cell lines. In BMS-S, when only one of the transgenes was flanked, the non-flanked transgene appeared to be silenced (Figure 25). However when the experiment was repeated in BMS-S5, ARS1 did not have the same effect; the unflanked transgene expressed at normal levels (Figure 26). This change is probably due to differences between the cell lines in their ability to silence transgenes. If ARS1 was responsible for silencing unflanked adjacent transgenes in BMS-S, it is possible that the factors involved in that silencing were either not present or not as prevalent in the BMS-S5. This may be why neighboring transgenes were no longer silenced in the older cell-line.

SAH response and MAR by transgene interactions. Mutant phenotypes have been observed frequently in cultured cell lines (DeMars, 1974; Siminovitch, 1976; Holliday et

al., 1996), but, in many cases changes in gene activity are due to epigenetic silencing rather than genetic mutations (Compere and Palmiter, 1981; Holliday et al., 1996).

Silenced genes in cell-lines or differentiated tissues of multicellular organisms are frequently methylated, and silencing may or may not be maintained by methylation. Of the cell lines tested with SAH, silencing was most active in BMS-S3. We found that, in BMS-S3, treatment with SAH derepressed non-flanked 35S::*GUS* expression more than in other cell-lines. For luciferase there was no significant increase in expression for either MAR-flanked or non-MAR events in BMS-S3. Nevertheless, in BMS-S4 and BMS-S5, the SAH treatment did increase luciferase expression, indicating that in these cell lines silenced copies of 35S::luciferase were methylated. Results with SAH in BMS-S4 and BMS-S5 were much more variable than in BMS-S3, however, suggesting that the capacity for maintaining genes in a silenced state may also have changed over time. Larger sample sizes would have been needed to determine in more detail how the effect of SAH treatment varied in older cell lines. However, the analysis of the pooled data clearly indicates a MAR by transgene effect.

MAR by transgene by copy-number interactions. In plants, multiple copies of a transgene usually result in silencing of the gene, via one or more poorly defined mechanisms commonly referred to as homology-dependent silencing (Jorgensen, 1990; Matzke and Matzke, 1995; Dorer, 1997). In BMS-S3, I found that increasing transgene copy number can increase expression levels in a manner similar to the effect of *Adh1* 5' MAR. As mentioned previously, *Adh1* 5' MAR shifts expression from the lower peak to the upper peak of the bimodal curve (at least in BMS-S and BMS-S3)(Figure 12A and 18A). This

same type of shift in expression levels also occurred in the GUS treatments without MARs when the DNA dose was increased from 1x to 100x (Figure 18B).

One explanation for the higher expression levels seen at the 100x DNA dose may be an interaction between the multiple copies of the transgenes that blocks silencing. However, this type of pairing has normally been associated in Drosophila with position effect variegation (Dorer and Henikoff, 1997). Another possibility is that, in this cell line, limiting levels of silencing factors may be titrated by the high transgene copy number, thereby increasing the probability that at high copy number, some of the transgenes will be active.

At the 100x DNA dose, the effects of MARs on either luciferase or *GUS* expression was masked. The *Adh1* 5' MAR had no effect on expression of *35S::GUS* when introduced with a MAR-flanked 35S::*BAR* vector. However, when a MAR-flanked 35S::luciferase vector was added in *trans*-, the MAR increased 35S::*GUS* expression for both the 1x and 100x doses. The effect of MAR-flanked 35S::*GUS* on 35S::luciferase expression was negligible by comparison. Because MARs flanking 35S::*GUS* only increased expression when introduced in combination with MAR-flanked 35S::*BAR* and MAR flanked 35S::luciferase and not with MAR-flanked 35S::*BAR* alone, interactions must be taking place between MARs in *trans*-, allowing the higher copy number of MARs to have an effect on 35S::*GUS* expression. This non-autonomous synergistic effect of MARs was seen also in the 2 x 2 experiment described in Section 4.2.1.

In general, when luciferase and *GUS* are introduced together, both transgenes are either expressed or silenced (Figure 19). In BMS-S and BMS-S3 at least, the expression distribution for both transgenes was bimodal. The bimodality could arise if there are two

different states of the cell or "epitypes" at the time when stable transcription states are established (Pillus and Rine, 1989; Loo and Rine, 1995). Each cellular epitype appears to act on both transgenes simultaneously to determine their expression states. At the 1x DNA dose, flanking both transgenes with MARs increases the likelihood that both transgenes will be expressed, independent of the cellular epitype. Cellular epitypes could be dictated by cell lineage, by cell age or by the stage of the cell cycle. Flanking MARs may increase the threshold level of factors that may be required for silencing to be established.

A recent study in *Drosophila* described a *lacZ* reporter under the control of a GAL4 UAS that also contained a Polycomb response element (PRE) (Cavalli and Paro, 1998). This construct also contained the *white* gene as a selectable marker. Because of the PRE, *white* gene expression was weak and variegated. However, when the transcriptional activator GAL4 was induced from a separate construct driven by a heat shock promoter, the Polycomb group (PcG) complex was displaced, and both *lacZ* and the *white* gene were expressed. When GAL4 was induced in embryos, the active state persisted and was maintained through many mitoses during somatic development. When GAL4 was induced during larval development, however, the derepression was temporary. This experiment illustrates how different epigenetic states of the cell determined by a balance of activating and silencing factors, can affect establishment and maintenance of stable gene expression patterns. In BMS cells, there may be a similar competition between activating and silencing factors that determine or establish the transcription state of an introduced transgene. On the one hand, activating factors that could interact with MARs might include proteins such as *trithorax* orthologs, transcriptional activators, or histone acetylases, whereas, on

the other hand, silencing proteins could include *polycomb* or SIR orthologs and histone deacetylases.

4.3 Other MAR Experiments

In previous sections of this chapter, I described various factors that influence expression of transgenes in BMS cells. In this section, I describe a variety of experiments that attempted to address other factors that might influence the efficiency of transformation. These include the effects of linking the selectable marker and reporter on the same plasmid, linearizing the plasmids prior to transformation, alternately flanking the reporter and/or the selectable marker with a MAR element, and changing the distance between the flanking MAR elements. Finally, I describe two unrelated experiments, one of which attempted to more precisely determine the sequences responsible for the *Adh1* 5' MAR effects on transgene expression. The other experiment demonstrates the accessibility of the *Adh1* 5' MAR to *trans*-activating factors *in vivo*.

4.3.1 Linking the selectable marker and reporter

Transformations using T-DNA and Agrobacterium obviously will have the selectable marker and reporter genes linked after integration into the genome. This is also the case for direct DNA transfer by particle gun bombardment. When a selectable marker and a reporter gene are on separate plasmids, both genes are typically linked after integration (Czernilofsky et al., 1986; Huang and Dennis, 1989). Because of this, I wanted to compare MAR-flanked and unflanked vectors, where the reporter and selectable maker were either on separate plasmids or in *cis*- on the same plasmid.

Figure 28 shows the vectors used and the results of the stable transformations with those vectors. Three comparisons were made, each in a different line of BMS. Figure 28A shows a comparison for ARS1-containing vectors. The histogram on the left summarizes expression level data obtained when the reporter (luciferase) and the selectable marker (*BAR*) were shot on separate plasmids. The histogram on the right summarizes data obtained with luciferase and *BAR* linked together in *cis*- and flanked with ARS1. Figure 28B is analogous to Figure 28A, except that *Adh1* 5' MAR was used instead of ARS1 flanking the transgenes, and BMS-S3 was used as the cell line. In Figure 28C, the downstream *Adh1* 5' MAR was replaced with the *Adh1* 3' MAR. Figure 28D summarizes data obtained from unflanked transgenes introduced in *cis*- or *trans*- in BMS-S5.

Because of changes in the cell lines, it is difficult to make general conclusions from these data. For ARS1, there was essentially no change between introducing the transgenes in *cis*- or in *trans*- in BMS-S (Figure 28A). This means that the plasmids in *trans*- most likely form concatamers upon integration and therefore behave similarly to the plasmid where both transgenes are already linked in *cis*-. However, the unflanked transgenes and the *Adh1* MAR flanked transgenes displayed a much higher average expression level in BMS-S3 or BMS-S5 when introduced in *cis*- (Figure 28B-D). This effect is possibly caused by the proximity of two 35S enhancer sequences adjacent to 35S::luciferase in the *cis*-vector. If this is the case, however, then ARS1 may suppress this effect, in contrast to the *Adh1* MARs.

These conflicting results are also confounded by the different degrees of transgene silencing in each cell line. In BMS-S, 35S::luciferase may have been more susceptible to

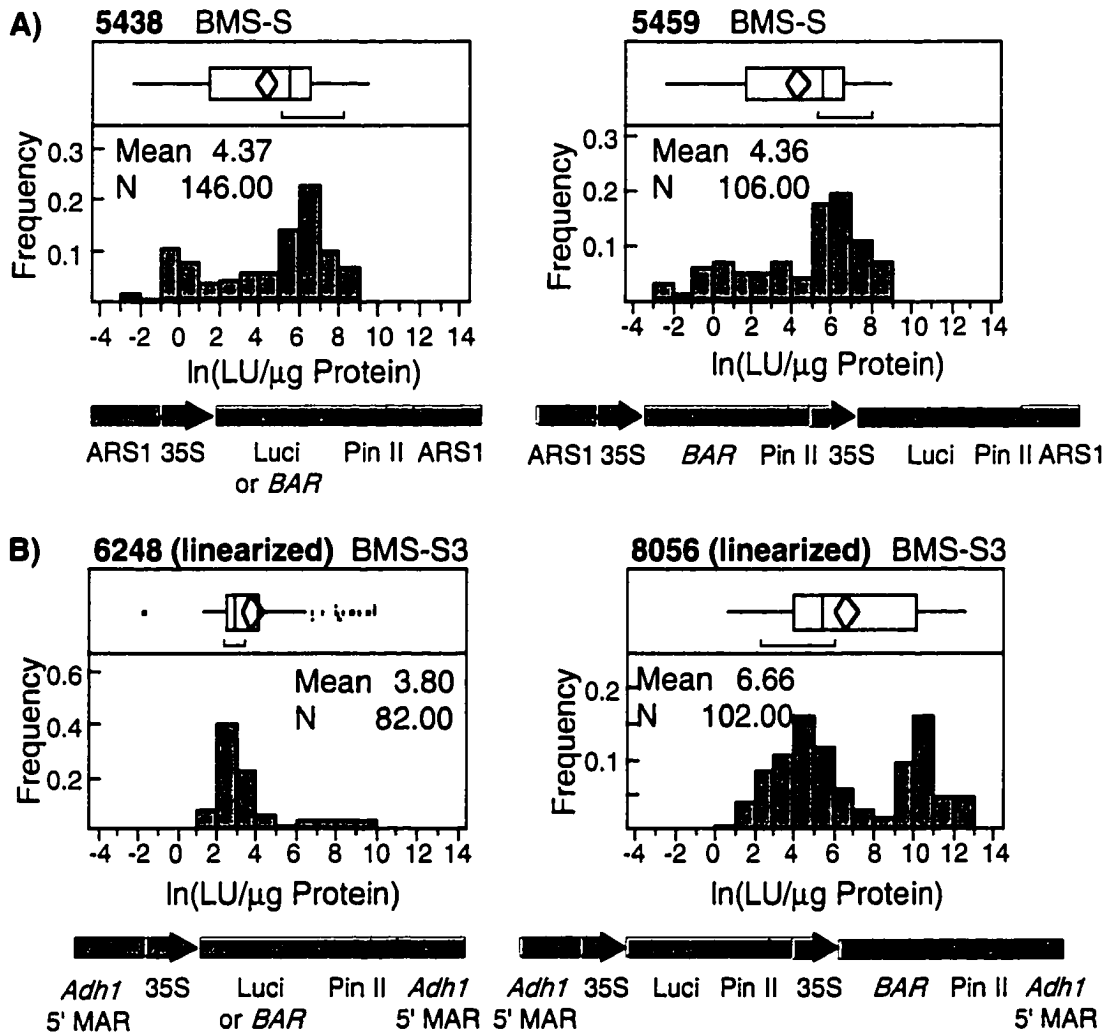

Figure 28. Comparison of co-transformation of transgenes in *cis-* vs. *trans-*. Luciferase and *BAR* were introduced in *trans-* on two separate plasmids or in *cis-* on a single plasmid. Plasmids were linearized as indicated before transfection by digesting with *Alw*NI, which cuts once in the vector backbone. All assays were done on the single-well luminometer except for those in BMS-S5 (D) which were assayed on the multiwell luminometer.

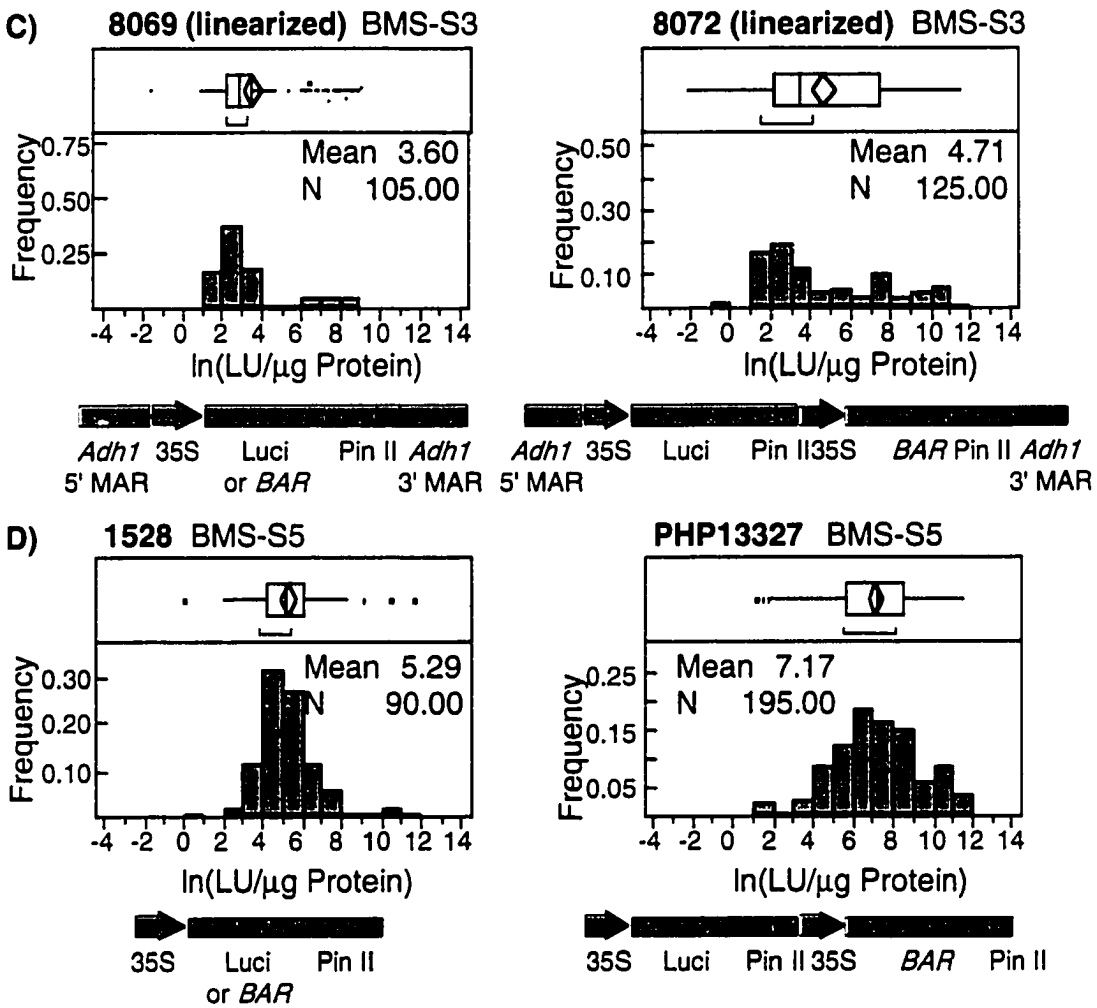

Figure 28. continued

silencing than in the other two cell lines. In this case, having the two genes linked would make little difference. However, in BMS-S3 and BMS-S5, if 35S::luciferase was less susceptible to silencing, then expression of the selectable marker in *cis*- may serve to keep the chromatin domain open and active, which could increase average levels of 35S::luciferase expression.

4.3.2 Linearized vectors

In several experiments, I tried linearizing the plasmids before bombardment. Because the transgene can occupy a significant proportion of the plasmid, there is a high likelihood that the breakpoint required for integration will occur within the transgene and interrupt its expression. In an attempt to increase the likelihood of recombination occurring outside the transgene, plasmids were linearized at restriction sites that fall outside of the transgene prior to transformation.

All experiments with linearized plasmids were done in BMS-S3. Figure 29 shows the results for two comparisons of circular versus flanked plasmids. In both cases, linearizing the vectors resulted in a reduction of the average level of expression. Although linearizing the vectors may have increased the chances that recombination took place at the ends of the linearized plasmid, it probably also increased the chances that the DNA was degraded after it entered the cell. Linearizing the DNA could also have made the DNA more susceptible to damage such as shearing. When particles are prepared for bombardment, the DNA is sonicated along with the tungsten beads. Thus, degradation of the DNA after entering the cell and shearing during particle preparation may each have contributed to the

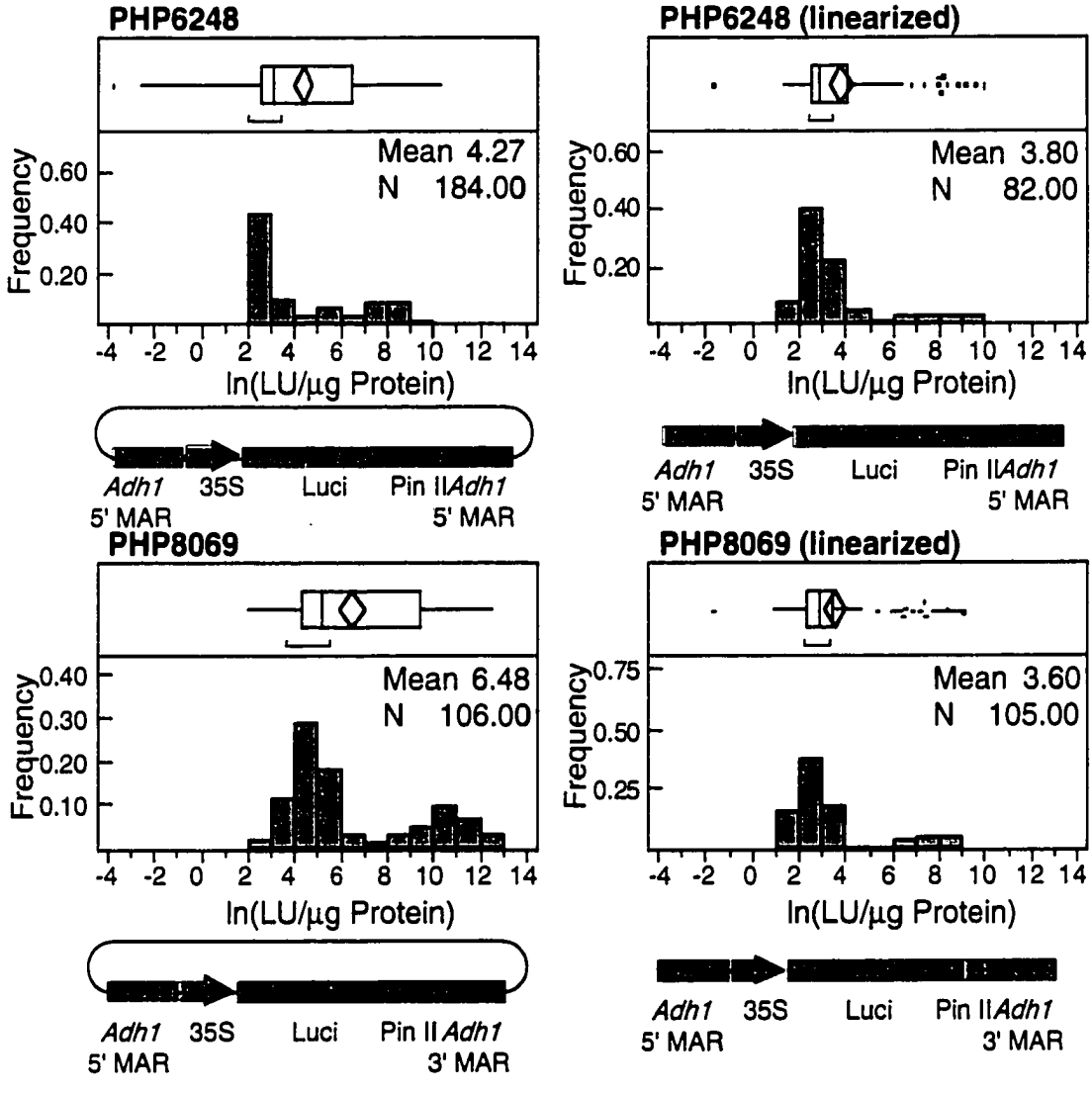

Figure 29. Supercoiled verses linear plasmids for input DNA. Plasmids were used supercoiled or linearized by digesting with *Alw*NI which cuts once in the vector backbone.

observed decrease in expression levels. Another possibility is that supercoiling of the template prior to integration helps the MARs exert their effect on establishing the transgene transcription state.

4.3.3 Distance between MARs

Another experiment that was done using linearized vectors examined the effect of increasing the distance between the flanking MARs. The distance between MARs in native chromatin typically varies between 5 kb and 200 kb. However, active genes are most frequently found in the smaller loops under 10 kb (Gasser and Laemmli, 1987). To test the effect of increasing the distance between the MAR elements, I inserted regions of lambda DNA between the *pin II* terminator and the MAR downstream of the transgene. The distance between the MARs in PHP6248 is approximately 3.6 kb. The two vectors containing lambda DNA increased the distance between the MARs to 6.5 kb in one (PHP8119) and 8.9 kb in the other (PHP13328). Increasing the distance between MARs beyond 8.9 kb made the plasmids too unstable in the strains of *E. coli* I had available.

Figure 30 shows that the effect of increasing the distance between the MARs was negligible. The 6.5 kb loop increased the average expression a small amount, but the increase was not significant. The 8.9 kb loop resulted in a decrease in the average expression. It is likely that this decrease was due to the factors mentioned previously that would affect linearized plasmids (i.e. higher susceptibility to degradation inside the cell and higher susceptibility to shearing during preparation for transformation). Even without these factors, it is likely that these constructs would have had few differences in their effect. Natural

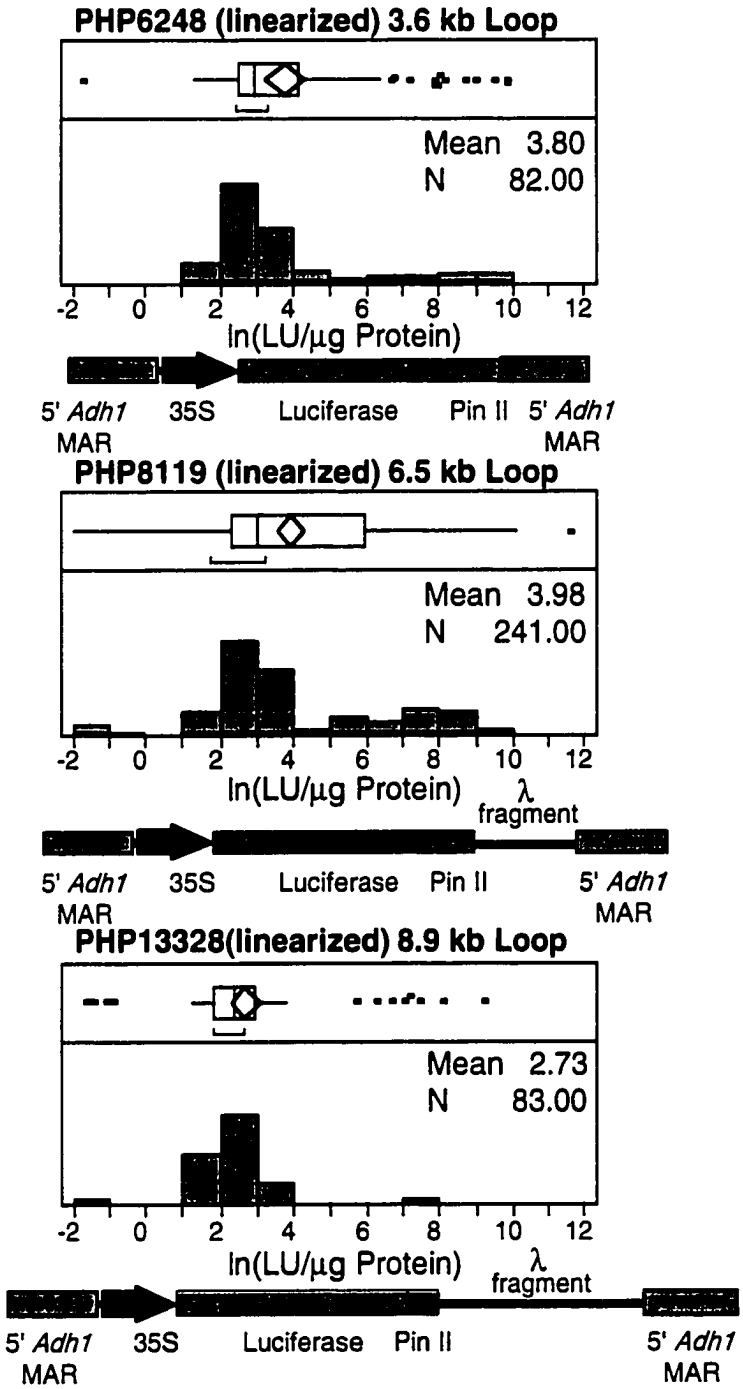

Figure 30. Results from increasing distance between MAR elements. The distance between MAR elements was increased by adding lambda DNA after the terminator and before the 3' MAR.

loop sizes in chromatin vary from 5 to 200 kb, a 40-fold difference. The increase from 3.6 kb to 8.9 kb is only 2.5-fold and still is not over the 10 kb size, where less active genes are thought to be found (Gasser and Laemmli, 1987).

4.3.4 Are *Adh1* 5' MAR effects on transgene expression conferred by transposable elements or by flanking sequences?

The *Adh1* 5' MAR contains two transposable elements, one inserted inside the other, that make up nearly half of the MAR sequence (Avramova et al., 1998; Zoya Avramova and Alex Tikhonov, personal communication). These sequences have a high degree of homology to miniature inverted-repeat transposable elements (MITEs) (Figure 31). Another example of a MAR containing transposons is the transformation booster sequence (TBS) from *Petunia hybrida*. This sequence, which can increase transformation frequencies in petunia, tobacco, and maize, contains a MAR element that has strong homologies to a variety of retroviral elements (Galliano et al., 1995). Martienssen (1996; 1998) and Matzke et al. (1996) have speculated that transposons play a role in gene silencing and imprinting phenomena in plants. In an attempt to determine if the MITEs in the *Adh1* 5' MAR were responsible for their effects in BMS, I constructed 35S::luciferase vectors flanked by fragments derived from the *Adh1* 5' MAR that either span the MITEs or the flanking sequence (Figure 31). Because the MITE region is about half the length of the *Adh1* 5' MAR, I doubled up the isolated MITE region to approximate the original MAR's length (Figure 2T-U). I also did the same for the vector flanked by non-MITE region of the *Adh1* 5' MAR (Figure 2V-W). The results of introducing these vectors into BMS-S5 are shown in Figure 32.

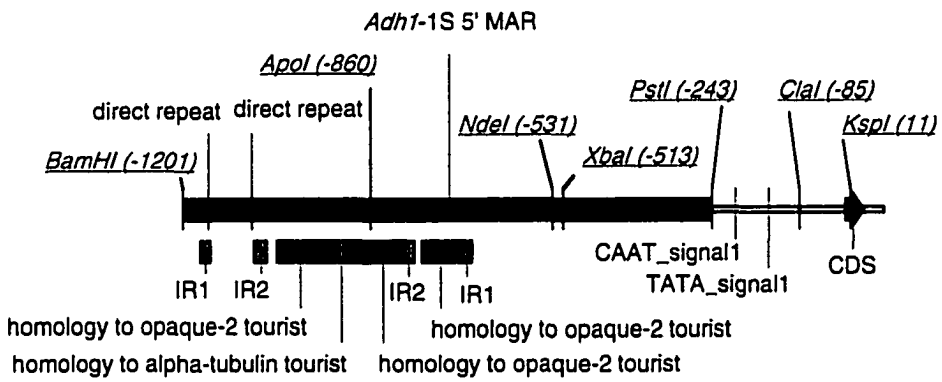

Figure 31. Map of the *Adh1* 5' region showing the locations of MITEs. Regions with homology to previously discovered MITEs are indicated (Bureau and Wessler, 1992). The region from -1201 to -680 was used for the MITEs containing vectors and from -680 to -243 was used for the non-MITEs vectors.

There did not appear to be any significant difference between the 2xMITE$^-$ and the 2xMITE$^+$ vectors. The average expression levels were essentially the same. Unfortunately, in BMS-S5, the *Adh1* 5' MAR had no effect on transgene expression levels, so it is difficult to make any conclusion regarding the lack of effect with the 2xMITE$^-$ and 2xMITE$^+$ vectors. If this experiment had been done earlier, when transgene silencing was more prevalent, it is possible I could have elucidated whether the sequences responsible for preventing transgene silencing in the *Adh1* 5' MAR resided within the MITEs or in the flanking sequences.

4.3.5 The *Adh1* 5' MAR is accessible to *dam* methylase

Dam methylase is a prokaryotic enzyme that methylates the adenine in the sequence GATC (Brooks et al., 1983). When introduced into yeast, *dam* methylase preferentially methylates its recognition sequence in active chromatin (Hoekstra and Malone, 1985;

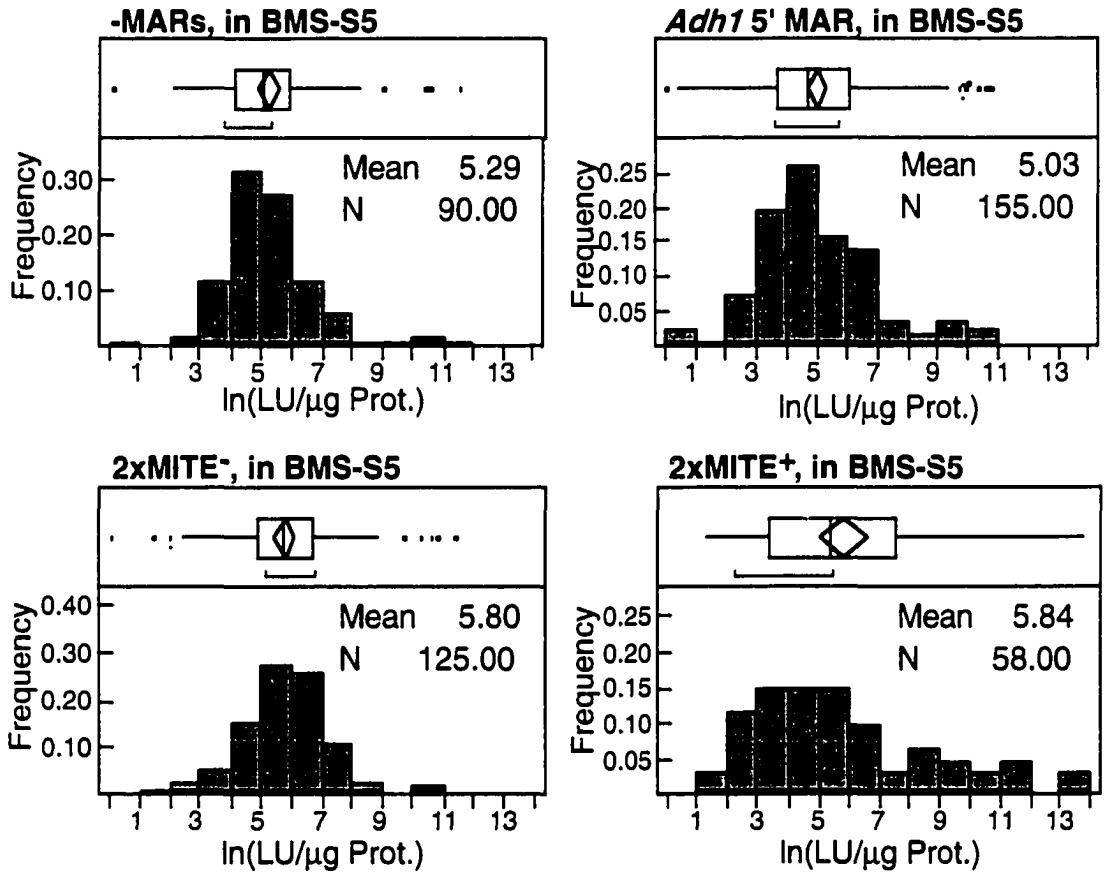

Figure 32. Attempt to determine if *Adh1* 5' MAR effects can be localized to MITEs. See text for more details of the experiment. There is essentially no difference in the mean expression levels between the 2xMITE$^-$ (PHP13396) and 2xMITE$^+$ (PHP13394) vectors in BMS-S5. It is possible that there would be a difference in cell lines with greater silencing potential, such as BMS-S.

Singh and Klar, 1992). Inactive chromatin is inaccessible to the enzyme. Because I wanted to determine if the *Adh1* 5' MAR was accessible to soluble factors inside the nucleus of BMS cells, I transformed BMS with *dam* methylase. To control expression of *dam* methylase in BMS, which I discovered was lethal (data not shown), I used an inducible system developed by Roth et al. (in prep.,see Section 3.6). After inducing *dam* methylase in BMS,

DNA was cut with the methylation sensitive isoschizomers *Dpn*I, *Dpn*II, and *Sau*3A and probed with the *Adh1* 5' MAR. A Southern blot of the results is shown in Figure 33. In the absence of *dam* induction, the *Adh1* 5' MAR hybridizes with high molecular weight DNA in both the uncut lane and the *Dpn*I lane, indicating that the GATC sequences near the *Adh1* 5' MAR are not normally methylated in BMS. In the *Dpn*II and *Sau*3A lanes, the *Adh1* 5' MAR probe hybridizes with a low molecular weight band, indicating that the DNA was purified adequately for restriction enzyme digestion. In the DNA extracted after *dam* induction, the *Adh1* 5' MAR probe hybridizes with a low molecular weight band in the *Dpn*I lane, indicating that *dam* methylase was able to methylate the *Adh1* 5' MAR region *in vivo*. This indicates that the *Adh1* 5' MAR must reside in open chromatin, which is consistent with the idea that the *Adh1* 5' MAR interacts with trans-acting factors *in vivo*.

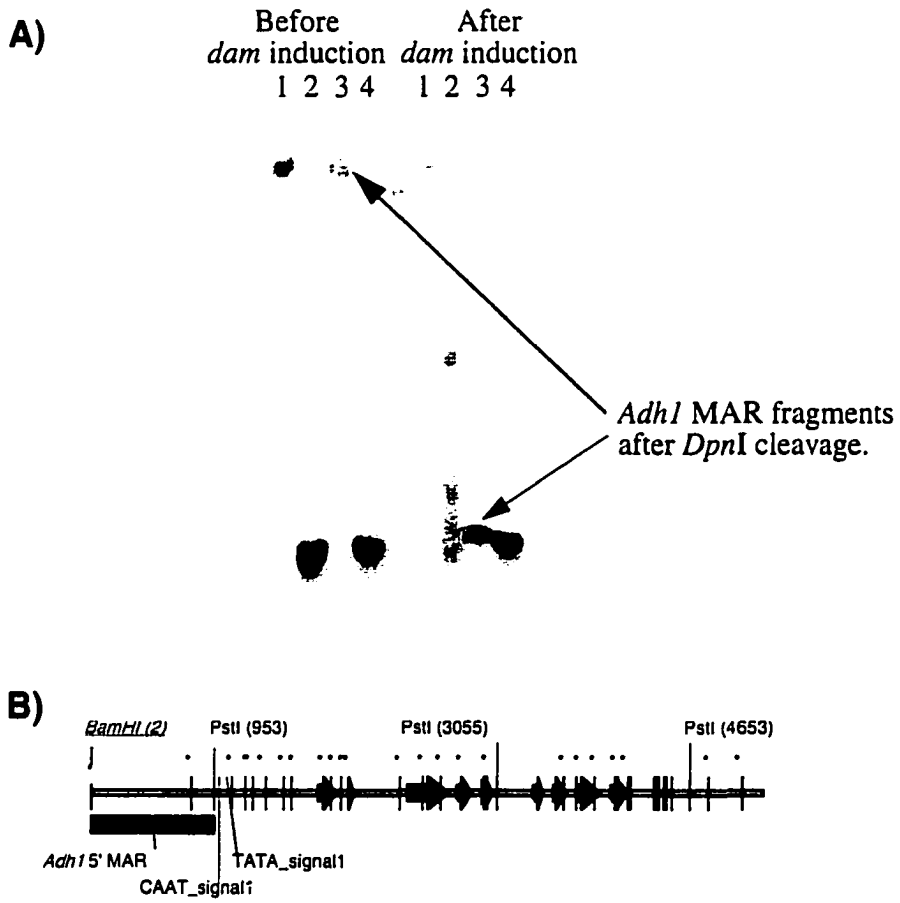

Figure 33. Southern showing accessibility of *Adh1* 5' MAR to *dam* methylase. A) Autoradiograph of Southern blot. Lane 1 is uncut DNA, lane 2 is *Dpn*II digest (cuts unmethylated GATC), lane 3 is *Dpn*I digest (cuts only methylated GATC), and lane 4 is *Sau3A*I digest (cuts regardless of methylation of GATC). When *dam* methylation is induced, a band appears in lane 3 indicating that the sites surrounding the *Adh1* 5' MAR were methylated. B) Restriction map of the maize *Adh1* gene, including the 5' MAR. Asterisks mark *Sau*3AI sites; numbers in parentheses represent nucleotides and hatched arrows indicate exons.

CHAPTER V. GENERAL CONCLUSIONS

5.1 Conclusions

Conservation of MAR properties between closely and distantly related species suggest that they must be an important component of the chromatin structure. The maize genome is highly repetitive, and all single copy regions studied so far are flanked by MAR elements (Avramova et al., 1995). Additionally, the placement of MAR elements is highly conserved. The *Adh1* regions of maize and sorghum show near identity in the order of genes and the placement of MAR elements flanking those genes (Avramova et al., 1998). This is true even though the sequences of the MARs are poorly conserved between the two species. Therefore the function of the MARs must be important. Also, if MARs were not important for the function of the genome, they would not be present so universally in eukaryotic cells.

Although the presence and location of MAR elements is conserved, I still can not state precisely what their function is. In my work on MARs, I have found that: 1) different MARs can have different effects on establishing transgene expression states which cannot necessarily be predicted from their *in vitro* properties (Section 4.1); 2) that the same MAR element can have different effects depending on the age of the cell line it is introduced into (Section 4.2); 3) one role of MARs may be to maintain stable patterns of transgene expression, either silent or expressed (Section 4.2); and 4) MARs can even have a silencing effect on the chromatin outside the gene they flank (Section 4.2).

Initially, I wanted to find out if MARs could insulate a transgene from position effects and increase the likelihood of obtaining a transgenic plant with a transgene expressing at

an optimum level. To test this, I decided to use a BMS callus system where I could analyze a large number of events. Then, using the data from callus, I hoped I could apply that knowledge to plants. The prognosis, however, is more complicated than just flanking your favorite gene with a good MAR element and transforming it into maize plants. My data has shown that a better understanding is needed of the context of the genome that our transgenes are introduced into. In particular, we need to know more about why the genome is arranged the way it is. We know that MARs flank endogenous genes, but why and how particular combinations of MARs allow genes to function properly *in vivo* is still unclear. Although I found that the *Adh1* 5' MAR can influence whether a transgene is active or silent, and if it stays that way, this seems to be the result of many interacting factors, and not some intrinsic property of the MAR sequence *per se*. MARs may not function to insulate genes but to signal various regulatory elements that a gene is nearby. In this way, perhaps, they help guide the proper proteins into position for ensuring appropriate gene regulation. Other studies have also shown that additional DNA sequences that interact with MARs are often required for proper transgene expression (Bonifer et al., 1994; Yu et al., 1994; Chinn and Comai, 1996; Huber et al., 1996).

If I had more time, there are still some experiments I would like to have done. Because I found that the effect of the *Adh1* 5' MAR differed in the various cell lines, it would have been interesting to see if the *Mha1* 5' MAR would have had an effect in one of the other BMS lines. It is possible, for example, that as the effect of the *Adh1* 5' MAR decreased in older BMS cells, the *Mha1* 5' MAR might have started to have an effect. This would have provided more evidence that MARs may both mediate and respond to different epigenetic

states. It would also be interesting to test if the *Mha1* 5' MAR could have an effect on the expression of GUS. Because there appears to be a MAR by transgene interaction, perhaps the *Mha1* 5' MAR could increase the average expression of *GUS*.

5.2 Application

MAR elements will not be useful in future transgene studies, unless the context in which they are to be used is understood. My results have indicated that there are MAR by transgene interactions, so MARs and transgenes must first be tested empirically to see if they function well together. As indicated earlier, other studies have demonstrated that additional elements may be required for proper transgene expression (Bonifer et al., 1994; Yu et al., 1994; Chinn and Comai, 1996; Huber et al., 1996). It will be important in studies of MARs in whole plants to determine when and where the MAR element has an effect. Typically, matrices and MARs have been isolated from leaf tissue. MARs selected in this way may have stronger effects on transgene expression in leaves than in other tissues.

More molecular studies such as those by Avramova et al. (1996) on the make up of natural chromatin and new microscopic studies showing the location and movement of DNA, RNA and proteins in the nucleus (see review Lamond and Earnshaw, 1998) are needed to understand how genes function in the nucleus. When we have identified the factors that are important to gene expression, then we can better mimic them when we try to introduce transgenes.

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IMAGE EVALUATION
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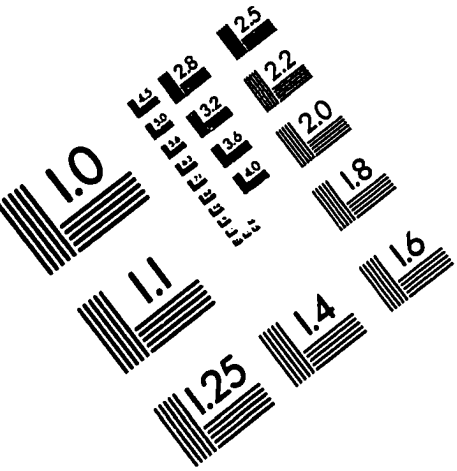

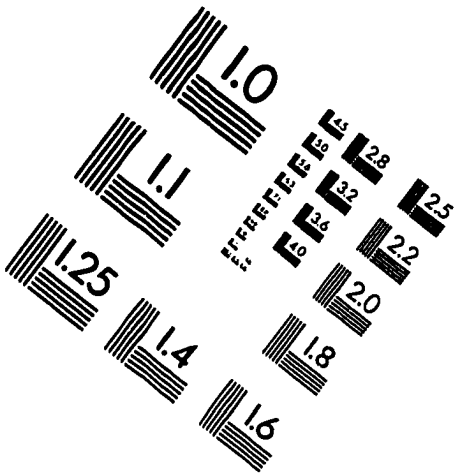

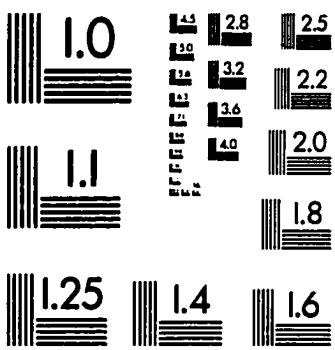

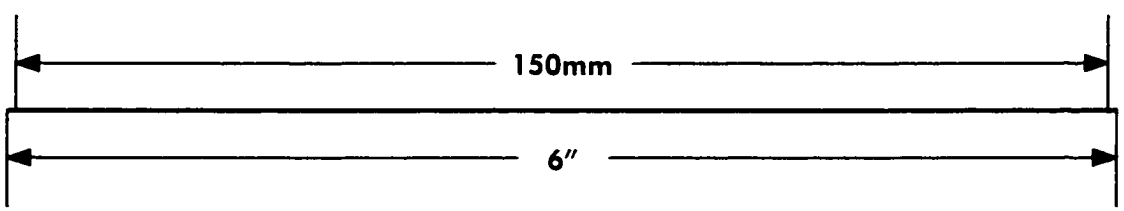

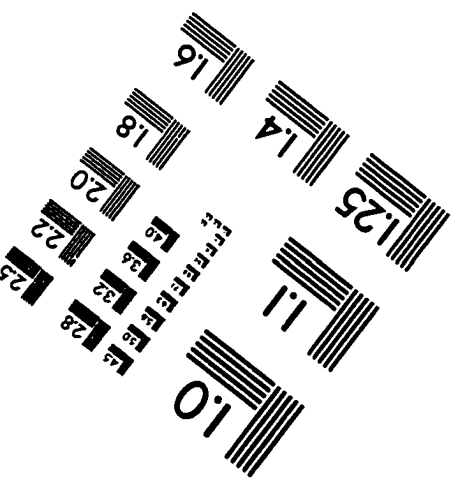

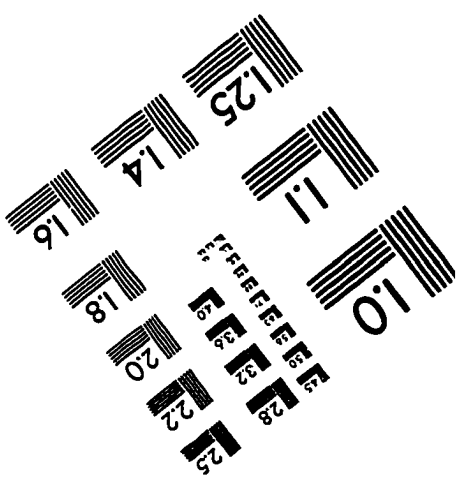

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